GENE-EXPRESSION ANALYSIS OF ACID INVERTASE AND SUCROSE-PHOSPHATE SYNTHASE IN MAIZE

By

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Invertase-deficient seed mutants in maize, *miniature1* (*mn1-1*) and several EMS-induced *mn1* allelic mutants, particularly *mn1-89*, were characterized to determine the relationship of the *Miniature1* (*Mn1*) seed locus to invertases and the relationship of invertase activity to seed size, seed weight, and an anatomical discontinuity (the gap) between pedicel and developing endosperm. *In situ* localization for the protein and/or RNA transcripts of the sucrose-metabolizing enzymes, invertase and sucrose-phosphate synthase (SPS), was done to gain a better understanding of sucrose movement/transport in developing kernels. The use of kernel culture technique and a suspension-cultured cell system was made to investigate the effects of sugars on the *mn1-1* seed mutant phenotype and sugar-modulated gene expression of sucrose-metabolizing enzymes, respectively. Finally, cell- and tissue-specific expression of SPS in maize leaf and kernel was studied using *in situ* immunolocalization for the protein.

Collective evidence supports a hypothesis that the *Mn1* locus encodes a cell wall-bound invertase, CWI-2, which is required for normal development of endosperm and maternal cells in the pedicel. The interruption of sugar transport into an endosperm due to the lack of invertase at the kernel base, as in the *mn1-1* seed mutant, caused a considerable reduction of seed weight (i.e., sink strength), up to ~70 to 80% of the wild type, and led to a gap formation between pedicel and endosperm. The *mn1-1* seed mutant was also associated with the secondary effects such as up- and down-regulation of SPS and SS proteins, respectively. The *mn1-89* mutant was leaky as it showed ~6% of wild type invertase activity and nearly normal seed size and weight. The invertase activity in this mutant was sufficient to arrest the gap formation, and SPS and SS protein levels were similar to those of the wild type. Thus, although a large proportion of invertase activity (~90%) was dispensable, the levels of invertase below a threshold value (~10%), played a rate-limiting role in controlling both the sink strength of the endosperm and the stability of maternal cells in the pedicel of a developing kernel. In addition, the results show that both soluble and cell wall-bound forms of invertase were coordinately regulated in various genotypes during the kernel development.

In vitro cultures of the *Mn1* and the *mn1-1* kernels on sucrose, fructose, or glucose, have shown the wild type and the mutant seed phenotypes, respectively. The mutant kernels did not show a restoration to the normal wild-type seed phenotype upon culture on hexose sugars. Similarly, the levels of invertase activities in the extracts from *Mn1* and *mn1-1* kernels grown *in vitro* were generally similar to those from *in planta*. Crude extracts from cob tissue which support *in vitro* culture of kernels were examined for invertases, SS isozymes and SPS by immunoblot analyses. Although there was no invertase activity/protein, the last two enzymes were detectable in cob extracts.

Coordinate regulation of both soluble and CWI forms of invertases was also seen in maize suspension-cultured cells. Furthermore, the data from suspension-cultured cells on sucrose-depleted medium showed a time-dependent coordinate loss of both the invertase isozymes, CWI-1 protein and cell wall-bound invertase (*incw1*) transcripts. After 48 hr incubation on sucrose-depleted medium, invertase activities (both soluble and the cell wall-bound) and the CWI-2 protein were extremely low. Yet, there was an induction of a new *incw1* transcript of slightly larger size than the normal *incw1* RNA. Significance of this new transcript which is presumably not translated, is unknown.

This study has also included experiments on SPS in both photosynthetic (leaf) and nonphotosynthetic (developing kernel) tissues. SPS protein was immunolocalized to both bundle sheath (BS) and mesophyll (M) cells of maize leaves. In young leaves, SPS was predominant in the BS cells, whereas in mature leaves it showed nearly equal levels in both BS and M cells. A cell-type-specific response was also seen in light and dark treatments, implying that SPS protein in BS cells may play an important role in sucrose synthesis in both photosynthetic and starch turn-over reactions. In addition, SPS protein in developing endosperm was localized in both basal endosperm cells and in the embryo. The developmental profile of SPS in endosperm was in remarkable parallel with invertase activity/protein present in kernel base. It is possible that sucrose breakdown and resynthesis occur in the basal endosperm cells.

CHAPTER 1
INTRODUCTION

Sucrose is the primary disaccharide and a preferred form of sugar for long-distant transport in most higher plants. It plays a pivotal role as a carbon source for energy production and storage reserves during plant growth and development. Sucrose is synthesized by the coupled actions of sucrose-phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP) or sucrose synthase, and hydrolyzed by invertase or cleaved by sucrose synthase (SS). These sucrose-metabolizing enzymes are generally present together in the same sink tissues to metabolize sucrose and determine an appropriate carbon partitioning in those tissues (Geigenberger and Stitt, 1991).

Invertase is present in two forms, cell wall-bound and soluble forms, in higher plants and microorganisms examined thus far. Each of the two forms of invertase has several isoform genes to form a small family (Jaynes and Nelson, 1971; Unger et al., 1994; Shanker et al., 1995; Taliercio et al., 1995; Xu et al., 1996). The function of cell wall-bound invertase (CWI) is believed to be involved in phloem unloading (Eschrich, 1980), and to establish an appropriate sucrose gradient between source and sink regions of a plant (Roitsch et al., 1995). It is conceivable that the lack of this enzyme will considerably influence carbon partitioning and ultimately plant growth and development. In this regard, the *mn1-1* seed mutant, first characterized by a ~80% loss of seed weight and a gap formation between pedicel and endosperm (Lowe and Nelson, 1946) is of special interest because it lacks both soluble and cell wall-bound forms of invertase in a developing kernel (Miller and Chourey, 1992). The lack of invertase activity in the *mn1-*

1 seed mutant presumably leads to the transient accumulation of sucrose and degeneration of placento-chalazal cells, i.e., a gap formation, in that region (Miller and Chourey, 1992). Consistent with this hypothesis are the data that sucrose concentration is higher in the pedicel and endosperm of this mutant, as compared to wild-type kernels (Shannon et al., 1993). However, it is not yet known whether the *Mn1* seed locus is a regulatory gene or a structural gene.

Although sucrose transport in the developing kernel has been studied for over two decades, it is still unclear how the sucrose is unloaded and metabolized in a developing seed. It is proposed that sucrose is first hydrolyzed in the pedicel and the hydrolyzed products (fructose and glucose) are taken-up by the basal endosperm cells in a passive manner (Shannon, 1972; Shannon and Dougherty, 1972; Porter et al., 1985; Griffith et al., 1987). It is also known that a certain level of sucrose may enter endosperm without hydrolysis in the pedicel (Cobb and Hannah, 1986; Schmalstig and Hitz, 1987; Felker, 1992). Histochemical staining for invertase activity shows that the enzyme is localized in the kernel base, including the pedicel (Doehlert and Felker, 1987), reflecting that sucrose and its hydrolyzed products in the pedicel might enter simultaneously into the extracellular space of basal endosperm cells.

Sucrose-phosphate synthase (SPS) is a key enzyme for sucrose biosynthesis. The enzyme is cytosolic and present in both photosynthetic and non-photosynthetic tissues (Bruneau et al., 1991; Reimholz et al., 1994). In photosynthetic tissues, carbon fixation occurs in chloroplasts, and is later exported as triose-phosphate into the cytosol. SPS in the cytosol plays an important role in controlling the rate of sucrose biosynthesis (Kalt-Torres et al., 1987; Rocher et al., 1989). Since C4 plants have dimorphic cell types in leaves, namely mesophyll (M) and bundle sheath (BS) cells, the location of SPS in maize leaves, a C4 plant, has been controversial. Furbank et al. (1985) showed that the SPS

activity is predominant in M cells, whereas Ohsugi and Huber (1987) found SPS localized in both M and BS cells. In non-photosynthetic tissue, SPS activity in potato tubers is regulated in a manner similar to the enzyme in the leaves (Reimholz et al., 1994). In the developing kernel, Chourey et al. (1993) have reported that *Sps* RNAs are restricted to basal endosperm. To gain insight into sucrose biosynthesis in maize leaves (photosynthetic tissue) and sucrose resynthesis in developing endosperm (nonphotosynthetic tissue), localization of SPS at the cellular level is essential.

Sugars regulate a number of plant genes (Sheen, 1990; 1994; for review: Koch, 1996, refs therein), including sucrose-metabolizing enzymes (for invertase: Roitsch et al., 1995; Xu et al., 1996; for SPS: Hesse et al., 1995; Weber et al., 1996; for sucrose synthase: Koch et al., 1992). Moreover, there are contrasting responses of the two isoform genes (*Shrunken1* [*Sh1*] versus *Sucrose synthase1* [*Sus1*]; *Invertase1* [*Ivr1*] versus *Invertase2* [*Ivr2*]) to the exogenous sugars (Koch et al., 1992; Xu et al., 1996). However, very little is known about the regulation of two CWI isoform genes, *incw1* (Shanker et al., 1995) and *incw2* (Taliercio et al., 1995) in maize suspension-cultured cells.

The primary objectives of this study are as follows: (1) to determine the relationship of the *Miniature1* seed locus to invertase and the subsequent relationships of invertase activity to seed size/weight, and the gap formation between pedicel and endosperm; (2) to test the effects of sugars on the *mn1-1* seed mutant phenotypes and to analyze sucrose-metabolizing enzymes in the cob tissues under *in-vitro* growth conditions; (3) to investigate possible bases for the coordinate control of the two forms of invertases in developing kernels and in suspension-cultured cells; (4) to determine cellular level localization of CWI (CWI-1 and CWI-2) and SPS in various tissues of the plant.

CHAPTER 2
LITERATURE REVIEW

Invertase

Characterization of Invertase

Invertase (β-fructofuranosidase, EC 3.2.1.26) is one of the key enzymes in sucrose metabolism. It catalyzes irreversible hydrolysis of the disaccharide sucrose into its monosaccharide components fructose and glucose: Sucrose + H_2O → Fructose + Glucose. The enzyme has been conventionally classified, based on its optimal pH, into three classes: acid (pH 4.5-5.4), neutral (pH 7.0), and alkaline (pH 7.5-8.0) invertase. Of these three classes, acid invertase has been found in a wide array of higher plant species and microbes, suggesting that it may be present in all organisms. Neutral and alkaline invertases are thought to be nonglycosylated cytoplasmic polypeptides (Copeland, 1990; Chen and Black, 1992). Neutral invertase is an octameric protein in carrot, whereas alkaline invertase is a homotetramer in carrot (Lee and Sturm, 1996) and soybean (Chen and Black, 1992). Neutral and alkaline invertase activities are found to be more active in mature tissues, where acid invertase is less active (Ricardo and ap Rees, 1970). However, there is evidence that all three forms of invertases may be present together in the same tissues (Morell and Copeland, 1984; Masuda et al., 1988; Ranwala et al., 1991). Since these invertase isoforms have different subcellular locations and unique biochemical

properties, they may independently regulate sucrose metabolism, translocation, and storage (Chen and Black, 1992).

A common feature of all organisms with acid invertase is the presence of two forms, soluble and cell wall-bound. The native molecular masses of soluble invertase proteins, located in the vacuole, are remarkably varied among plant species. In carrot, for instance, the molecular masses of soluble invertase are ~56 kDa in cultured cells while there are two isoforms of 58 and 52 kDa in seedling (Sturm and Chrispeels, 1990). Nevertheless, the molecular masses of native invertase isoforms in maize show apparent differences ranging from 750 kDa to over 900 kDa (Doehlert and Felker, 1987). The soluble enzyme is proposed to be a heterodimer in mung bean (Arai et al., 1992) and carrot (Unger et al., 1994). Cell wall-bound invertase (CWI) proteins, ionically bound to the cell walls and extractable with high salts, have molecular masses of 63 kDa in carrot (Sturm and Chrispeels, 1990) and 40 kDa in maize (Doehlert and Felker, 1987). The CWI protein appears to be a monomer in tobacco crown-gall cells (Weil and Rausch, 1994). Both forms of invertase have been purified to/or near homogeneity in many plant species and have been characterized for their biochemical properties (Masuda and Sugawara, 1980; Faye and Ghorbel, 1983; Singh and Knox, 1984; Krishana et al., 1985; Doehlert and Felker, 1987; Lauriére et al., 1988; Fahrendorf and Beck 1990; Sturm et al., 1995). Generally, the CWIs have an acidic pH for optimal activity, and a lower Km for sucrose than the soluble forms. In addition, the pI values for CWI isozymes are alkaline, while soluble invertases have more acidic pI values.

Both soluble and cell wall-bound forms of invertase are N-linked glycoproteins (Doehlert and Felker, 1987; Sturm, 1991), with high mannose and complex glycans. Glycosylation may promote subunit aggregation, as is the case with yeast invertase, resulting in large native forms of invertase (Chu et al., 1983).

It has been suggested that CWI may play an important role in phloem unloading and carbon partitioning (Eschrich, 1980), osmoregulation (Meyer and Boyer, 1981; Porter et al., 1987), gravitropism (Wu et al., 1993), wounding, and pathogen infection (Matsushita and Uritani, 1976; Sturm and Chrispeels, 1990). In sink tissues, it may hydrolyze sucrose to maintain a steep sucrose concentration gradient between source and sink regions of a plant (Eschrich, 1980; Miller and Chourey, 1992) and establish sink strength for storage or sink tissue development (Roitsch et al., 1995). CWI is usually found at high levels in rapidly growing tissues with a high demand for hexose, such as the extending zones of root tips (Duke et al., 1991), tap roots, internodes, leaves (Eschrich, 1980), or endosperms (Tsai et al., 1970; Doehlert and Felker, 1987; Miller and Chourey, 1992). High soluble invertase activity is often observed in young seedling, tuberous roots (Ricardo and ap Rees, 1970), and mature fruits (Elliott et al., 1993). The soluble invertase is involved in the regulation of hexose levels in mature tissues and in the mobility of vacuole-stored sucrose (Leigh et al., 1979; Unger et al., 1992).

Invertase-Deficient Mutants

Since invertase plays a pivotal role in sucrose catabolism and carbon partitioning, the interruption of invertase activity must have severe effects on plant growth and development. In this regard, the *miniature1* (*mn1*) seed mutant of maize, first described by Lowe and Nelson (1946), is characterized by a 80% seed-weight loss and a gap formation between pedicel and endosperm. Furthermore, Miller and Chourey (1992) found that the mutant lacked both soluble and cell wall-bound forms of invertase in the kernel base. Invertase deficiency as well as the seed phenotype are specific to the endosperm. It is believed that the gap formation in the mutant is the causal effect of the loss of invertase in the kernel base, causing the transient accumulation of sucrose and an early degeneration of placento-chalazal cells (i.e., a gap formation) in the pedicel. Consistent with this speculation are the data that sucrose concentration in the pedicel of the mutant is higher than that in the wild type (Shannon et al., 1993). Another invertase-deficient mutant, OH43, lacks invertase in maize primary root tips and can not utilize sucrose on sucrose-supplemented agar medium (Robins, 1958; Duke et al., 1991).

Molecular Cloning of Acid Invertase Genes

Both soluble and cell wall-bound forms of invertase have been cloned from many plant species, such as carrot (Sturm and Chrispeels, 1990; Ramloch-Lorenz et al., 1993; Unger et al., 1994), mung bean (Arai et al., 1992), tomato (Klann et al., 1992; Elliott et al., 1993; Sato et al., 1993), potato (Hedley et al., 1993), tobacco (Greiner et al., 1995),

Chenopodium rubrum (Roitsch et al., 1995), *Vicia faba* (Weber et al., 1995), *Arabidopsis* (Mercier and Gogarten, 1995), maize (Shanker et al., 1995; Taliercio et al., 1995; Xu et al., 1995), and grape berry (*Vitis vinifera*) (Davies and Robinson, 1996). In most cases, there is a unique set of both forms of invertase present in each tissue of a plant (Unger et al., 1994; Roitsch et al., 1995; Weber et al., 1995). Each of the two forms of invertase is known to have several isozymes to form a small family (Jaynes and Nelson, 1971; Unger et al., 1994; Roitsch et al., 1995; Weber et al., 1995; Shanker et al., 1995; Taliercio et al., 1995; Xu et al., 1995; Davies and Robinson, 1996). Unlike yeast, in which both forms of invertase are encoded by a single gene (Carlson and Botstein, 1982), the soluble and cell wall-bound forms of invertase in higher plants examined thus far are believed to be encoded by distinct genes.

A comparison of DNA or deduced amino acid sequences reveals that high homology or similarity is observed among the members of the same class from diverse taxonomic groups, whereas it is low between the classes (Unger et al., 1994; Weber et al., 1995). For instance, the deduced amino acid sequence of maize soluble invertase, *Ivr1*, has ~59 and 42% identity to carrot soluble and cell wall-bound forms of invertase, respectively (Xu et al., 1995). Similarly, maize CWI, *incw1*, has ~57 and 58.1% amino acid identity with tobacco and carrot CWI, respectively, and ~43.5% identity with carrot soluble invertase (Shanker et al., 1995). The derived amino acid sequences of invertase are pre-proenzymes with a signal peptide and N-terminal propeptide (Unger et al., 1994).

Ectopic Expression of Acid Invertase in Transgenic Plants

The constitutive expression of yeast-derived invertase in the apoplastic regions of tomato (Dickinson et al., 1991), *Arabidopsis* (von Schaewen et al., 1990), tobacco (von Schaewen et al., 1990; Sonnewald et al., 1991), and potato (Heineke et al., 1992) interrupts sucrose export from source leaves into the phloem cells of minor veins. Such interruption leads to accumulation of fructose, glucose, and certain amino acids, but a reduced level of sucrose in mature leaves. Consequently, there is increased osmolarity in leaf cells, accompanied by the decline of photosynthesis and stunted growth of the transgenic plants. These responses are associated with down- and up-regulations of Calvin-cycle and glycolytic enzyme activities, respectively (Stitt et al., 1990). In addition, the transgenic plants normally show symptoms, characterized by the development of greenish-yellow and green sectors in the same leaf. Such symptoms may progress basipetally and finally form chlorotic and necrotic lesions in mature leaves. Moreover, ultra-anatomical (or electron microscope) study reveals that the greenish-yellow regions with high CWI activity have arrested development of secondary plasmodesmata, which may prevent free movement of signal molecules among mesophyll cells, thus resulting in early leaf senescence (Ding et al., 1993). Interestingly, transgenic tobacco plants expressing vacuolar and apoplastic yeast-derived invertase show necrotic lesions similar to hypersensitive response (HR) caused by avirulent pathogens. These lesions are accompanied by elevated levels of defense-related gene transcripts, callose content, peroxidase activities, salicylic acid, and a subsequent systemic acquired resistant

(SAR) response. In contrast, the expression of a cytosolic yeast-derived invertase in tobacco plants with equally elevated levels of sugars yields neither a SAR response nor a decline of photosynthesis. Herbers et al. (1996) have suggested that hexose sensing occurs in secretory pathways to trigger the defense-related genes and repress photosynthetic genes in vacuolar and apoplastic transgenic plants.

Regulation of Acid Invertase Expression

Sugar-Modulated Expression of Acid Invertase

Recently, there is increasing evidence that a number of genes, including invertase, are metabolically regulated by sugars. For instance, a variety of studies involving sucrose and starch metabolism, or the storage protein in potato tuber, show that the genes encoding class I patatin (Roch-Sosa et al., 1989), potato inhibitor II (Johson and Ryan, 1990), ADP-glucose pyrophosphorylase (Müller-Röber et al., 1992), granule-bound starch synthase (Visser et al., 1994), and sucrose synthase (Salanoubat and Belliard, 1989) can be induced by elevated sucrose concentrations. In *Arabidopsis*, sucrose mimics the light induction of nitrate reductase gene transcript (Cheng et al., 1992); chalcone synthase transcription is also stimulated by sugars (Tsukaya et al., 1991). In maize, the two sucrose-synthase genes (*Sh1* and *Sus1*) (Koch et al., 1992) display contrasting responses to sugars (sugar-enhanced and -repressed). In addition, there are certain examples where sugars cause a repression of gene expression. In maize mesophyll

protoplasts, there are at least seven photosynthesis-related genes which are repressed in the presence of sugars (sucrose, glucose, or acetate) (Sheen, 1990, 1994; Jang and Sheen, 1994). Gene expression of chlorophyll a/b binding protein and *rbcS* in spinach leaves (Krapp et al., 1993), and α-amylase in rice suspension-cultured cells (Yu et al., 1991) are also reduced with the addition of sugars.

As for invertase expression in response to sugars, Kaufman et al. (1973) have shown that the *Avena* stem segments grown in the presence of sucrose revealed an increased level of invertase activity. Furthermore, such an effect is enhanced by the addition of gibberllic acid (GA), suggesting that the induction of invertase might provide the carbon source needed for GA-promoted growth. By adding glucose to the medium, photoautotrophic suspension-cultured cells of *Chenopodium rubrum* are shifted to mixotrophic growth, accompanied by elevated levels of CWI activity and transcript (Roitsch et al., 1995). This implies that the presence of CWI may establish a metabolic sink, other than the typical function of phloem unloading and carbon partitioning in a plant. In maize, the two invertase genes (*Ivr1* and *Ivr2*) encoding the soluble form of invertases are differentially regulated by sugars (sucrose and glucose) in excised root tips, such that *Ivr1* and *Ivr2* gene expression is enhanced in the absence and presence of sugars, respectively (Xu et al., 1996). Taken together, sugar molecules *per se*, particularly hexoses (glucose and fructose), may function as signals to trigger gene expression through kinase-mediated pathway(s) (Jang and Sheen, 1994). Consequently, the sugar-modulated genes might regulate metabolic pathway(s) to adjust sugar status or

carbon partitioning in response to sugar or environmental stimuli (Jang and Sheen, 1994; Özcan et al., 1996).

Other Factors: Wounding, Pathogen Infection, Phytohormones, and Endogenous Inhibitors

While undergoing environmental stress, such as wounding and pathogen attack, plants have evolved a capability to mount a defense response for survival. Since cells have a high hexose demand for active metabolism under these stresses, the increased acid invertase activity may provide a carbon source and enhanced photoassimilate flow into the cells, which are required for the defense response and recovery from damage (Matsushita and Uritani, 1974; Long et al., 1975; Billet et al., 1977; Callow et al., 1980; Sturm and Chrispeels, 1990).

Although plant growth and development are well controlled by endogenous phytohormones, several lines of evidence have shown that acid invertase activity is enhanced in expanding plant tissues with high levels of phytohormones (Morris and Arthur, 1984; Ishikawa et al., 1988) or under exogenous phytohormone treatments (Gordon and Flood, 1979; Howard and Witham, 1983; Miyamoto et al., 1993). A well-known case is the *Agrobacterium tumefaciens*-infected tobacco cells, which contain high levels of endogenous IAA, cytokinin, and acid invertase activity (Ishikawa et al., 1988). The elevated acid invertase activity may make more substrate for active metabolism during cell growth (Ricardo and ap Rees, 1970).

Acid invertase activity can be inhibited by its proteinaceous inhibitors, which have been found in many plant species, such as tobacco (Weil and Rausch, 1994; Weil et al., 1994), potato (Pressey, 1968; Anderson et al., 1980; Bracho and Whitaker, 1990a, 1990b; Ovalle and Ewing, 1991), maize (Jaynes and Nelson, 1971), sweet potato (Pressey, 1968; Matsushita and Uritani, 1976), red beet, sugar beet (Pressey, 1968), and tomato (Pressey, 1994). The inhibitors are located either in the vacuoles of potato (Bracho and Whitaker, 1990a, 1990b) or extracellular space (cell walls) of tobacco (Weil et al., 1994). The inhibitors are generally co-purified with acid invertase proteins, resulting in acid invertase activity not always corresponding to immunological detection of the enzyme protein (Weil and Rausch, 1994).

Sucrose-Phosphate Synthase

Characterization of SPS

Sucrose-phosphate synthase (SPS, EC 2.4.1.14), first discovered by Leloir and Cardini (1955), a key enzyme for sucrose biosynthesis, catalyzes the conversion of UDP-glucose and fructose-6-phosphate into UDP and sucrose-phosphate. Ultimately, sucrose is formed after the removal of orthophosphate group from sucrose-phosphate catalyzed by sucrose-phosphate phosphatase (SPP). SPS is cytosolic and present in both photosynthetic (Furbank et al., 1985; Ohsugi and Huber, 1987) and nonphotosynthetic (Hawker, 1985; Geigenberger and Stitt, 1991; Guy et al., 1992; Reimholz et al., 1994;

Weber et al., 1996) tissues of higher plants. During photosynthesis, carbon fixation occurs in chloroplast and is later exported as triose-phosphate into the cytosol, where the presence of SPS serves as the key enzyme for sucrose biosynthesis (Stitt and Quick, 1989). It have been suggested that SPS plays an important role in controlling the flux of carbon into sucrose since photoassimilate exports and plant growth rate are positively correlated with SPS activity (Kalt-Torres et al., 1987; Rocher et al., 1989). SPS activity shows a diurnal fluctuation during day/night cycles in spinach and maize (Sicher and Kremer, 1985; Kalt-Torres et al., 1987; Ohsugi and Huber, 1987). Such enzyme activity alteration is regulated by metabolic factors (Doehlert and Huber, 1983, 1985; Huber and Huber, 1992; Weiner et al., 1992) and protein phosphorylation (Huber et al., 1989a; Siegl et al., 1990; Huber and Huber, 1991; Huber and Huber, 1992), but is not due to a circadian clock. However, SPS protein shows no significant change under diurnal (day/night) cycles in mature maize leaf (Bruneau et al., 1991). SPS protein has been partially purified from maize leaves and potato tubers. The molecular mass of SPS is 124.8 or 133.5 kDa in potato tuber (Reimholz and Stitt, 1994).

Regulation of SPS Activity

Although SPS activity reveals a diurnal fluctuation during day/night cycles, the enzyme activity is regulated at several levels. First, it is regulated by metabolic factors (i.e., allosteric regulation), such as glucose-6-phosphate (an activator) and Pi (an inhibitor) in both photosynthetic (Weiner et al., 1992) and non-photosynthetic tissues

(Reimholz et al., 1994). These factors may bind to allosteric sites of the protein and may rapidly affect the enzyme activity. This mechanism is often termed as "fine" control (Huber and Huber, 1992).

Second, SPS is regulated by protein phosphorylation (covalent modification). In the light, SPS is dephosphorylated and becomes active (Huber and Huber, 1991); in contrast, the enzyme is phosphorylated and becomes inactive under dark conditions (Huber et al., 1989a; Siegl et al., 1990; Huber and Huber, 1991). Phosphorylation and dephosphorylation events are controlled by a SPS-protein kinase and a type 2A protein phosphatase (Stitt and Quick, 1989; Huber and Huber, 1992). This mechanism is often termed "coarse" control (Huber and Huber, 1992) since these protein modifications are slow as compared to those which are controlled by the metabolites. The major regulatory phosphorylation site(s) have been identified in several plant species, such as Ser-158 in spinach (McMichael et al, 1993) and Ser-162 in maize (Huber et al., 1995). All sequences available to date show a homologous seryl residue, reflecting that it might be important for recognition by protein kinase (Huber and Huber, 1996). A synthetic peptide corresponding to the amino acid sequence of the phosphorylated region in spinach was used to isolate SPS antiserum, which could discriminate the phosphorylated or dephosphorylated state of the SPS protein (Weiner, 1995). Both protein phosphorylation and allosteric regulation are interdependent. For instance, glucose-6-phosphate is an activator for SPS activity and protein phosphatase, but it is an inhibitor for the SPS-

protein kinase; in contrast, Pi is inhibitor for SPS activity and the protein phosphatase, but it is an activator for the SPS-protein kinase.

Third, SPS activity is also dependent on the protein content or developmental stage. SPS activity/protein is higher in spinach mature leaves than in young leaves (Walker and Huber, 1989). Similarly, high level of SPS RNA and protein are also found in source leaf of spinach (Klein et al., 1993).

Fourth, SPS activity is also correlated to plant species or genetic variability. Three groups of plant species are classified based on the biochemical characteristics of the SPS protein. The class I group shows a marked light sensitivity to SPS, as evidenced by an increase in Vmax; the class II shows no effect on Vmax; and the class III species is light insensitive (Huber et al., 1989b). Moreover, Rocher et al. (1989) have analyzed eight maize genotypes with various growth rates; the results demonstrated that the SPS activities varied among these genotypes and this variation was positively correlated with their respective growth rates.

Molecular Cloning of SPS

At the molecular level, SPS has been cloned from maize (Worrel et al., 1991), spinach (Klein et al., 1993), sugar beet (Hesse et al., 1995), and rice (Sakamoto et al., 1995). The deduced amino acid sequences show that they have molecular masses ranging from 120-135 kDa (Huber et al., 1994), and share a high identity (Hesse et al., 1995). The high conserved amino acid sequence appears in the regions of the substrate binding sites and protein modification sites by kinase/phosphatase in all known cDNA clones. In

sugar-beet leaf, *Sps* transcripts are up- and down-regulated by exogenous glucose and sucrose treatments under light condition (Hesse et al., 1995). Similarly, the level of *Sps* transcripts in seeds of *Vicia fava* is also up-regulated by desiccation and exogenous hexose (Weber et al., 1996). Heterologous expression of maize SPS gene in tomato causes an elevated levels of SPS, an increase of sucrose and a decrease of starch in leaves. It confirms a previous hypothesis that SPS plays a critical role in the regulation of carbon partitioning in plants (Worrel et al., 1991).

CHAPTER 3
A CRITICAL ROLE OF MAIZE CELL WALL-BOUND INVERTASE IN NORMAL DEVELOPMENT OF ENDOSPERM AND MATERNAL CELLS OF THE PEDICEL

Introduction

Invertase (β-Fructofuranosidase, EC 3.2.1.26), one of the key enzymes in sucrose metabolism, catalyzes the essentially irreversible hydrolysis of sucrose to fructose and glucose. The enzyme (acid invertase), in most higher plants examined thus far, contains two forms, soluble and cell wall-bound. Each of the two forms of invertase is known to have several isoforms to form a small family (Jaynes and Nelson, 1971; Unger et al., 1994; Shanker et al., 1995; Taliercio et al., 1995; Xu et al., 1996). Unlike yeast in which both forms of invertase are encoded by a single gene (Carlson and Botstein, 1982), soluble and cell wall-bound forms of invertase in higher plants have been isolated from various plant species and turn out to be encoded by distinct genes (Sturm and Chrispeels, 1990; Elliot et al., 1993; Ramloch-Lorenz et al., 1993; Unger et al., 1994; Greiner et al., 1995; Roitsch et al., 1995; Shanker et al., 1995; Taliercio et al., 1995; Weber et al., 1995; Xu et al., 1995).

Furthermore, a comparison of soluble with cell wall-bound forms of invertase reveals that each tissue in a plant has a unique set of genes for the two isoenzymes. In addition, the cDNA clones or their deduced amino acid sequences of the two forms of invertase appear to have higher homology or identity with members of the same class from diverse taxonomic groups (Unger et al., 1994; Weber et al., 1995). In maize, for instance, the deduced amino acid sequences of the *incw1* cDNA clone, a cell wall-bound

invertase (CWI) encoding a CWI-1 protein, isolated from suspension-cultured cells (Shanker et al., 1995), shares 59.1% sequence identity with the carrot CWI cDNA (Sturm and Chrispeels, 1990), whereas it shares only 44.7% identity with the maize soluble invertase clone (Xu et al., 1995). Likewise, another CWI clone, *incw2* encoding a CWI-2 protein, isolated from immature kernel base (Taliercio et al., 1995), shares 70.5% sequence identity with CWI-1.

Soluble invertase, located in the vacuole (Lauriére et al., 1988; Stommel and Simon, 1990), is believed to regulate hexose levels in the cytosol and the utilization of sucrose stored in the vacuoles (Ricardo, 1974; Unger et al., 1992). The cell wall-bound invertase, ionically bound to extracellular cell walls (Doehlert and Felker, 1987), is believed to be related to phloem unloading to establish an appropriate carbon partitioning between source and sink regions of a plant (Eschrich, 1980; Unger et al., 1992; Roitsch et al., 1995).

It is conceivable that the interruption of sucrose loading from the source tissues into the vascular system or unloading from the terminal ends of the vascular system into the sink tissues must cause a dramatic effect on normal growth and development of a plant. In this regard, there is increasing evidence that supports the physiological role of CWI. For instance, the ectopic expression of a yeast-derived invertase in the extracellular cell walls of tobacco, *Arabidopsis*, and tomato plants, interrupts sucrose export from the source tissues and leads to an accumulation of carbohydrates and inhibition of photosynthesis in mature leaves, accompanied by morphological alteration and stunted growth (von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991). In addition, the transgenic plants also have a decrease of the Calvin-cycle enzymes and an increase of glycolytic enzymes (Stitt et al., 1990).

The *miniature1* (*mn1*) seed mutant, first described by Lowe and Nelson (1946), is characterized by an 80% loss of seed weight and a gap formation between pedicel and endosperm. Miller and Chourey (1992) found that this mutant lacks both soluble and cell wall-bound forms of invertase in the kernel base; moreover, there is genetic evidence that it is an endosperm trait. The lack of invertase in the mutant causes an over-accumulation of sucrose in the pedicel (Shannon et al., 1993), perhaps leading to an osmotic imbalance in this region and subsequently withdrawal of pedicel from endosperm and/or degeneration of placento-chalazal in the pedicel. Thus, the gap formation between pedicel and endosperm is believed to be the causal effect of invertase deficiency in the *mn1* seed mutant (Miller and Chourey, 1992).

The aim of this study is to characterize *mn1-1* and several newly isolated EMS-induced allelic *mn1* seed mutants to address the relationship of the *Miniature1* (*Mn1*) seed locus to invertase and the subsequent relationships of invertase activity to seed size, weight (or sink strength) and the gap formation between pedicel and endosperm. Collective evidence presented here supports a hypothesis that the *Mn1* seed locus is a structure gene, encoding a CWI-2 protein. Although a substantial level of invertase activity was dispensable (~90%), there was a threshold value (~10%), below which the enzyme played a rate-limiting role in normal development of endosperm and the maternal cells of the pedicel. Moreover, both soluble and cell wall-bound forms of invertase were regulated coordinately in various genotypes and throughout kernel development examined.

Plant Materials and Methods

Plant Materials

All genetic stocks representing homozygous *Mn1* and *mn1-1* (the reference allele) and EMS-induced *mn1* alleles, *mn1-74*, *mn1-82*, *mn1-83*, *mn1-84*, *mn1-88*, and *mn1-89*, were in W22 genetic background. In addition, homozygous *Mn1* stock was also in Pioneer 3165 inbred-line background. The strategies for the isolation of EMS-induced mutants have been described previously (Briggs et al., 1965; Chourey and Schwartz, 1971). Because the isolation was based on *mn1-1* seed phenotype, all isolated seed mutants were indistinguishable from the *mn1-1* mutant, except for *mn1-89*, which revealed a unique seed phenotype with intermediate seed size, pale anthocyanin pigmentation, and no papery pericarp. The *mn2* seed mutant was of an unknown genetic background. All plants were grown in the greenhouse or field; the temperature was controlled between 28 and 32°C in the former with a normal diurnal (day/night) pattern. Kernels at various developmental stages were harvested, frozen immediately by adding liquid N_2, followed by storage in -80°C until use, or fixed in formalin acetic alcohol (FAA) for immunolocalization or *in situ* hybridization studies.

Genetic Crosses for Determining Seed Weights

F_2 ears were obtained by selfing the *Mn1/mn1-1* and *Mn1/mn1-89* F_1 heterozygotes. Several F_2 ears showing a 3:1 segregation for the wild type and either the homozygous recessive *mn1-1* or *mn1-89* kernels were obtained. Homozygous mutant kernels from several F_2 ears were pooled and weighed against the corresponding wild-type kernels with *Mn1* allele. Similarly, two sets of testcrosses with (1) *Mn1/mn1-89* x *mn1-1/mn1-1* (male parent) and (2) *Mn1/mn1-1* x *mn1-89/mn1-89* (male parent) were

made. Both sets led to BC1 ears with seeds of two distinct seed sizes that segregated in a 1:1 ratio (see Table 3-2). Based on previous genetic analyses with these genotypes, we attributed the smaller seeds to endosperm genotypes as *mn1-89/mn1-89/mn1-1* and *mn1-89/mn1-1/mn1-1* in set (1) and set (2), respectively. The larger seeds in each set were due to the dominant *Mn1* allele.

Invertase Enzyme Assays

Frozen kernels were homogenized in extraction buffer in a 1:10 (w/v) ratio by using a chilled mortar and pestle. The extraction buffer used in the isolation of soluble invertase protein contained 50 mM Tris-maleate, pH 7.0, and 1 mM DTT (Doehlert and Felker, 1987). The homogenate was centrifuged at 14,000*g* for 10 min; the supernatant was removed for soluble invertase assays followed by 1 hr or overnight dialysis. The pellet was washed three times in extraction buffer followed by a final resuspension in extraction buffer containing 1 M NaCl in a 1:2 (w/v) ratio. The salt suspension was vortexed in an Eppendorf mixer (model 5432; Eppendorf Corp., Madison, WI) for 30 min at 3°C and subsequently centrifuged at 14,000*g* for 10 min. The supernatant was dialyzed against extraction buffer without NaCl at 3°C for 1 hr or overnight and used as cell wall-bound invertase fraction in enzyme assays, as described previously (Tsai et al., 1970; Miller and Chourey, 1992).

SDS Immunoblot Analysis

Crude protein extracts were prepared from frozen kernels as described above. A 0.25 volume of 4X SDS buffer was added into crude extracts to generate a final volume containing 1X SDS buffer (60 mM Tris, 2% SDS, 10% glycerol, 5% mercaptoethanol), followed by heating in boiling water for 3 min to denature proteins. One aliquot of the

crude extracts was used to determine protein content (Lowry et al., 1951). Denatured protein samples were separated on an SDS-polyacrylamide gel, according to Laemmli (1970). For gel blot analyses, proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) and treated according to the instructions provided with a Du Pont staining kit (New Renaissance). Briefly, the membrane was blocked in 5% nonfat dry milk in 10 mM PBS-T (PBS-Tween 20) for 1 hr. After two washes with PBS-T, each for 5 min, the membrane was incubated with CWI, SPS polyclonal antibodies, SS1, or SS2 monoclonal antibody. After the first wash for 15 min and four subsequent washes, each for 5 min, the membrane was incubated with the secondary antibody anti-rabbit immunoglobulin conjugated with horseradish phosphatase (Sigma) for 1 hr. After four washes, the membrane was treated with chemiluminescence reagent (Nel-100; Du Pont) for 1 min before developing. Polyclonal antibodies against carrot CWI and maize SPS proteins were kind gifts from Drs. A. Sturm (FMI, Basel, Switzerland) and T.A. Voelker (Calgene, Inc., Davis, CA), respectively. SS1 and SS2 monoclonal antibodies specifically recognize SS1 and SS2 proteins, respectively; there is no cross-reactivity with each other (Chourey et al., 1991b).

Immunohistochemical Localization

Freshly harvested kernels at various developmental stages were fixed in formalin acetic alcohol, dehydrated through tertiary butyl alcohol series, infiltrated in Paraplus (Fisher Scientific), embedded, sectioned, and immunostained essentially following the protocol described by Chen and Chourey (1989) (see Appendix), with the few exceptions noted below. In brief, to ensure the complete removal of tertiary butyl alcohol from infiltrated kernels, they were subjected to three changes of fresh liquid Paraplus, each for 2 to 3 hr, before embedding. Longitudinal sections were cut ~10 or 12 μm in thickness

by using a rotary microtome. Paraffin was removed from sections on slide in xylene and sequentially hydrated to 30% ethanol. After further washing in distilled water and PBS, slides were reacted with primary antibody or preimmune serum, as a negative control, for ~4 hr. The slides were then reacted with secondary antibody solution composed of biotinylated anti-mouse anti-rabbit immunoglobulin and streptavidin alkaline phosphatase. Immunolocalized signal of CWI was visualized using New Fuchsia chromogen (Dako Corp., Carpinteria, CA), resulting in a precipitate of fuchsia-colored end product at the site of the antigen.

In Situ Hybridization

cDNA inserts, ~2.2 kb size, corresponding to the *incw1* and *incw2* genes (Shanker et al., 1995; Taliercio et al., 1995), were isolated from maize suspension-cultured cells and kernel base, respectively. The pBluescript SK(+/-) vectors containing cDNA inserts were used in this study. Nearly full-length sense and anti-sense strands were labeled with digoxigenin-UTP using *in vitro* transcription reactions with T3/T7 RNA polymerase, according to the instructions provided with the DIG RNA Labeling Kit (Boehringer Mannheim). The hybridization essentially followed the protocol described by Marrison and Leech (1994), with the few exceptions noted below.

To eliminate RNase contamination, all solutions used prior to and during the hybridization were incubated with 0.1% (v/v) diethylpyrocarbonate overnight at 37°C before autoclaving. ProbeOn Plus slides (Fisher Scientific) were used in this study.

Sections were deparaffined and rehydrated through an ethanol series to water as described previously for immunolocalization. Pretreatment of hybridization included the followings: Sections were incubated in 0.2 M HCl for 20 min at room temperature, rinsed in water, followed by incubation in 2x SSC for 30 min. Subsequently, sections

were treated with 1 μg/ml proteinase K in 100 mM Tris (pH 8.0), 50 mM EDTA for 30 min at 37°C, followed by incubation in 2 mg/ml glycine in PBS for 2 min at room temperature to terminate proteinase K activity. Sections were refixed in 4% (w/v) paraformaldehyde/PBS for 20 min. Dehydration included treatment with 0.1% (v/v) acetic anhydride in 0.1 M triethanolamine-HCl, rinsed in PBS and dehydrated in an ethanol series to 100% alcohol. After drying briefly, sections were circled with a Pan-Pen marker (Research Products International Corp., Mount Prospect, IL) to prevent flooding of the hybridization mixture. Hybridization was carried out by the addition of hybridization mixture (sense or antisense + hybridization medium) on sections. The hybridization medium contained 50% (v/v) formamide, 10 % (w/v) dextran sulphate, 300 mM NaCl, 10 mM Tris-HCl (pH 6.8), 5 mM EDTA, 10 mM sodium phosphate, 10 mM DTT, 1 mg/ml tRNA (Sigma), and 0.5 mg/ml poly A^+ RNA (Sigma). Slides were incubated in a humid chamber at 56°C for 20 hr.

After hybridization, slides were incubated for 4 hr at 56°C in wash buffer, composed of 50% (v/v) formamide, 300 mM NaCl, 10 mM Tris (pH 6.8), 10 mM sodium phosphate, 5 mM EDTA. Non-specifically bound probe was removed by the incubation of slides with 20 μg/ml RNase A in NTE (500 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA) for 30 min at 37°C followed by 5x 10 min washes in NTE at 37°C and an additional incubation in wash buffer for 1 hr at 56°C. The final wash was in PBS overnight at 4°C.

Immunological detection of the hybridized probe was done essentially according to the manufacturer's instruction (Boehringer Mannheim). Briefly, slides were incubated for 45 min in 0.5 % (w/v) blocking reagent in PBS (Boehringer Mannheim), followed by 1% (w/v) BSA, 0.3% (v/v) Triton-X-100 in PBS for 45 min. Then, sections were treated with anti-digoxigenin-alkaline phosphatase conjugate in 100 mM Tri (pH 7.5), 150 mM

NaCl for 1 hr at room temperature. Unbound antibody conjugate was removed by washing slides 3x 20 min in 1% (w/v) BSA, and 0.3% (v/v) Triton-X-100 in PBS. Slides were then washed briefly in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$ and incubated in color development solution (0.34 mg/ml nitroblue tetrazolium chloride (NBT) and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$ for 3-16 hr, based on the amount of labeled RNA. Alkaline phosphatase activity leading to the (blue-purple) colored precipitation was stopped by incubation of slides in 10 mM Tris (pH 8.0) and 1 mM EDTA, followed by passing slides through an ethanol series to xylene. Finally, slides were mounted with Permout reagent.

Results

Invertase Activity During Kernel Development

Kernels at various stages were harvested and invertase activity was examined to determine the invertase activity profile during kernel development. As shown in Figure 3-1, the highest level of invertase specific activity was at 12 days after pollination (DAP), with a rapid reduction thereafter until a later stage 28 DAP. At this stage from 28 to 32 DAP, the enzyme activity remained relatively constant and contained ~ 25 to 30% of the highest level of enzyme activity. Of the two isoforms of invertase, the cell wall-bound form was predominant throughout developmental stages; it accounted for ~90% of total enzyme activity compared to soluble form (~10%). However, in the *mn1-1* mutant, both forms of invertase remained low to undetectable during 12 to 24 DAP period (Figure 3-1A).

The CWI protein was also examined using SDS immunoblot analysis. The polyclonal antiserum raised against carrot CWI showed no cross-reactivity to the soluble

form of invertase in both maize (data not shown) and carrot (Unger et al., 1992). The SDS immunoblot in Figure 3-1B showed CWI polypeptides in crude extracts prepared from the lower third of endosperm, throughout all developmental stages tested (Figure 3-1B, lanes 1, 3, 5, 7, and 9). However, the CWI polypeptide was low to undetectable from extracts prepared from the upper two-thirds of endosperm (Figure 3-1B, lanes 2, 4, 6, 8, and 10). Surprisingly, the CWI polypeptides in crude extracts of the pedicels and suspension-cultured cells revealed a slightly smaller size, estimated as ~68 kDa (p68) (Figure 3-1C, lanes 1 and 3, respectively), than that in endosperm, ~72 kDa (p72) (Figure 3-1C, lane 2). It indicates that the CWI polypeptide in the pedicel and suspension-cultured cells is another isoform distinct from the CWI in endosperm.

Gene-Dose Relationship of *Mn1* Locus with Invertase Activity and Protein

To better understand the genetic basis of the loss of invertase activity in the *miniature1* (*mn1-1*, the reference allele) seed mutant, the author was provided with six new *mn1* seed mutants, each of independent origin (see Plant Materials and Methods), induced by EMS mutagenesis. Of these six mutants, one mutant, *mn1-89*, is of special interest due to its unique phenotype and was analyzed in more detail. Table 3-1 shows the comparative levels of both cell wall and soluble forms of invertase activities in kernel extracts from homozygous *Mn1*, *mn1-89* and *mn1-1*, and the heterozygous genotypes obtained by reciprocal crosses among *Mn1* and *mn1-1*, *Mn1* and *mn1-89*, and *mn1-89* and *mn1-1* parents. There are at least three striking aspects noted below.

First, compared to homozygous *mn1-1* mutant, the homozygous *mn1-89* mutant showed a fourfold higher activity in both cell wall and soluble forms. However, both *mn1-89* and *mn1-1* mutants had only ~6 and 1.5% of total enzyme activity of normal wild type, respectively. Second, there is a near linear relationship of *mn1-89* with total

invertase activity in triploid endosperm genotypes between the combination of *mn1-89* and *mn1-1* alleles. The endosperm with three copies of the *mn1-89* allele had the highest level of invertase activity (~6%); however, zero copy had the least (~1.4%). Two and one copies of the *mn1-89* allele obtained by reciprocal crosses with *mn1-1* had 3.9 and 2.2% levels of normal wild-type activity, respectively. A similar relationship was also seen in the combination of *Mn1* with *mn1-89*, or *Mn1* with *mn1-1* alleles. Overall, three copies of the *Mn1* allele had the highest levels of activity, with a gradual reduction at two, one, and zero copies of the *Mn1* allele. In each set of experiments, CWI activities were closer to the expected linearity than the activities of soluble forms. And finally, the cell wall-bound and soluble forms of invertase appeared to be regulated coordinately in various genotypes. Particularly, in each case, the relative increase or decrease of cell wall-bound invertase activities was always associated with a corresponding change in levels of the soluble form.

To further determine whether the CWI protein in various genotypes of kernels also exhibits gene-dose relationship, crude extracts were prepared from whole kernels (Figure 3-2A) or lower third kernel bases (Figures 3-2B and -2C) in two sets of parents: (1) *Mn1* versus *mn1-1* (Figures 3-2A and -2B) and (2) *Mn1* versus *mn1-89* (Figure 3-2C) and the reciprocal hybrids between each set of parents. As shown in Figure 3-2, the p72 (CWI-2) polypeptide was readily detectable in all genotypes, except for the *mn1-1* mutant (Figures 3-2A and -2B) and the five newly induced mutant alleles (data not shown). CWI-2 was restricted to the lower third of kernel (Figures 3-2B and -2C), where the enzyme activity was enriched ~5- to 6-fold higher than that in whole kernel (data not shown). The p72 polypeptide in the *mn1-1* mutant remained low to undetectable even though we enriched invertase protein in extracts from kernel base (Figure 3-2B, lane 4). It is noteworthy that the crude extracts from the *mn1-89* homozygote had a faint band of

p72 protein (Figure 3-2C, lane 4), which was undetectable in the *mn1-1* mutant. This indicates that *mn1-89* is a leaky mutant. These differences between the two mutants are consistent with the enzyme activity data (Table 3-1) showing that the *mn1-89* mutant had 4-fold higher of CWI activity than did in the *mn1-1* mutant. The residual level of the invertase activity in the *mn1-1* mutant was below the detection limits of the immunoblot used in this study. Overall, these results illustrate that the band intensities of the p72 polypeptide are dependent on the copy number of *Mn1* allele, and are consistent with the enzyme activity in various genotypes.

Developmental Stability of the Pedicel and Seed Size/Weight

It is proposed that the gap between pedicel and endosperm in the *mn1-1* seed mutant is due to the loss of invertase in the mutant endosperm (Miller and Chourey, 1992). To better understand the genetic basis of this mutant, the *mn1-89* and *mn1-1* homozygotes, and their reciprocal hybrids were examined using the anatomical methods. As shown in Figure 3-3, the homozygous *mn1-89* parent and the hybrid with 2 copies of *mn1-89* allele (i.e., *mn1-89/mn1-89/mn1-1*, obtained by *mn1-89mn1-89* (female parent) X *mn1-1mn1-1*) showed no detectable gap formation between endosperm and pedicel at 12 DAP. In contrast, the hybrid with one copy of *mn1-89* allele (i.e., *mn1-89/mn1-1/mn1-1*, obtained by *mn1-1mn1-1* (female parent) X *mn1-89mn1-89*) showed a gap, and it was also indistinguishable in seed phenotype from the *mn1-1* homozygote (Figure 3-3C versus 3-3D). Thus, the early withdrawal of the pedicel is determined by the levels of invertase activity in an endosperm.

Figure 3-4 represents comparative seed phenotypes of various genotypes at maturity, particularly showing several gene doses of *mn1-89* allele. The homozygous *mn1-89* seed mutant (Figure 3-4, the second ear) displayed a slight reduction in seed size

compared to the wild type (Figure 3-4, the first ear). The reciprocal hybrid with two copies of *mn1-89* allele (Figure 3-4, the third ear) was slightly smaller in seed size than its parent homozygous *mn1-89* , but larger than the hybrid with only one copy of *mn1-89* allele (Figure 3-4, the fourth ear), which was indistinguishable from its parent homozygous *mn1-1* (data not shown). Thus, the reciprocal hybrids, with different endosperm genotypes of either two copies of *mn1-89* (obtained by *mn1-89mn1-89 X mn1-1mn1-1*) or one copy of *mn1-89* (obtained by *mn1-1mn1-1 X mn1-89mn1-89*) but the same embryo (diploid) genotype (*mn1-89mn1-1*), were readily distinguishable from each other. It indicates that the *mn1-89* allele is semidominant to *mn1-1*. The seed phenotype of the hybrid with two copies of *mn1-89* was closer to its maternal parent homozygous *mn1-89*; likewise, the hybrid with one copy of *mn1-89* (i.e., two copies of *mn1-1* allele) resembled its maternal parent homozygous *mn1-1*. In fact, in this case the latter hybrid phenotype was indistinguishable from homozygous *mn1-1* seed mutant. Interestingly, the intensity of anthocyanin pigment was also determined by the copy number of *mn1-89* allele. Perhaps, the carbon moiety of anthocyanin compounds in triploid aleurone layer of endosperm is also limited by the CWI activity, particularly, when the enzyme activity is below 6% of homozygous wild type.

To determine whether the distinct seed phenotypes are correlated with seed weights (i.e., sink strength), we examined F_2 generation ears that were segregating for homozygous *mn1-1* or *mn1-89* kernels along with wild type kernels on the same ear. Relative seed weights (Table 3-2), shown as percentage of normal wild type, were 36.4 and 77.3% for homozygous *mn1-1* and *mn1-89*, respectively. Appropriate testcrosses were also made (see Methods) to obtain kernels with one or two copies of the *mn1-89* allele that were segregating among wild-type kernels on the same ears. Relative seed weights for one or two copies of *mn1-89* allele were 43.5 and 70.8% of the wild type,

respectively. Overall, these seed-weight data are in good agreement with their corresponding seed size phenotypes (Figure 3-4).

Immunolocalization and *in situ* Hybridization of Cell Wall-Bound Invertase protein and RNA Transcripts in Kernel Sections

Invertase enzyme activity, as documented by histochemical staining, in developing kernels is restricted to the kernel base, including the pedicel, throughout developmental stages tested (Doehlert and Felker, 1987; Miller and Chourey, 1992). Our immunoblot analysis have now confirmed these data on the enzyme activity (Figures 3-1 and 3-2). To further determine the invertase location at the cellular level, immunolocalization and *in situ* hybridization were done in this study in various genotypes at 12 to 16 DAP. As shown in Figure 3-5, positive signals, as evidenced by an intense fuchsia-colored reaction product, were readily detectable in *Mn1* (Figures 3-5B, -5C, and -5D), but not in *mn1-1*, *mn1-89*, and preimmune-treated *Mn1* sections (Figures 3-5F, -5E, and -5A, respectively). The low levels of p72 protein in the *mn1-89* seed mutant, determined by immunoblot analyses (Figure 3-2C), was perhaps below the detection limits of this method. The positive signals seen in *Mn1* sections were predominantly limited to apoplastic region and to wall-in-growths of the basal endosperm transfer cells (Figures 3-5B, -5C, and -5D). A low-level signal was also detectable in the pedicels along upper vascular bundle regions of the *Mn1* kernels (Figures 3-5B and -5C). The staining intensity was consistent with the low levels of p68 protein in the pedicel extracts determined by immunoblot assays (Figure 3-1C). Another non-allelic mutant, *mn2,* showing a similar miniature seed phenotype, was also examined. A positive signal in *mn2* section (Figure 3-5G) was consistent with our data showing normal levels of invertase activity and p72 protein by using immunoblot assays (data not shown). Thus, the invertase deficiency was specific to the *mn1-1* seed mutant.

In situ hybridization analyses were done on the sections of kernels of various genotypes to localize *incw2* RNA at the cellular level. Positive signals upon hybridization with digoxigenin-labeled *incw2* antisense RNAs, as evidenced by the blue-purple end product, were seen in basal endosperm transfer cells of *Mn1* kernel sections (Figures 3-6B, -6C, and -6D); no such signal was seen on sections from the *mn1-1* kernels (Figure 3-6F). In contrast, there was no detectable signal in the *Mn1* kernel sections hybridized with *incw2* sense construct (Figures 3-6A). Kernel sections from the *mn1-89* mutant also yielded a positive *incw2* signal (Figure 3-6F), consistent with the previous data showing the mutant with ~6% of wild-type invertase activity (Table 3-1) and much reduced levels of the p72 protein (Figure 3-2). The miniature seed mutant, *mn2*, revealed nearly normal levels of *incw2* transcript at basal endosperm transfer cells (Figure 3-6G), consistent with its normal levels of CWI activity and protein (data not shown). However, unlike the immunolocalization tests, there was no hybridization signal in the pedicel of the *Mn1* kernel sections (Figures 3-6B and -6C). It is presumably because the CWIs in pedicel and endosperm are isoforms encoded by two distinct genes. Thus, the *incw2* probe, representing the kernel gene, specifically hybridized to the transcripts in the basal endosperm cells, and not to the transcripts in the pedicel. However, there was still no signal in the pedicel when the *incw1* clone was used as a probe. It is likely that the CWI transcripts in the pedicel were low to undetectable levels since the relative total invertase activity in the pedicel extracts was only ~20% of the homozygous wild-type activity in terms of whole kernel (data not shown). Furthermore, *in situ* localization for RNA was a less sensitive tool than the immunolocalization for protein, as judged by the relative levels of the signal intensities (Figures 3-5B versus 3-6B).

Discussion

Collective Evidence That *Mn1* Encodes a Cell Wall-Bound Invertase

Several lines of evidence presented here support a hypothesis that the *Mn1* seed locus encodes a CWI protein, CWI-2. On a genetic basis, three sets of lineage-related hybrids, *mn1-89/mn1-1*, *mn1-89/Mn1*, and *mn1-1/Mn1*, and their parents revealed a gene-dose relationship of *Mn1* locus with CWI enzyme activity (Table 3-1) and CWI-2 protein (Figure 3-2). Several loci of gene-enzyme relationships regarding starch biosynthesis have also shown similar linearity with their gene products (Nelson and Pan, 1995). In addition, of the six EMS-induced *mn1* mutants, five of them were phenotypically indistinguishable from *mn1-1* and showed low to undetectable levels of invertase activity and protein, as seen in the *mn1-1* mutant. Unlike the *mn1-1* mutant, which loses ~80% seed weight (Lowe and Nelson, 1946), the mutant, *mn1-89*, lost seed weight only by ~20% and appeared to be a leaky mutant at enzyme activity and protein levels (Table 3-1 and Figure 3-2). Overall, all EMS-induced *mn1* mutants, isolated according to *mn1-1* seed phenotype, are defective in CWI-2 invertase activity/protein. Such a situation is similar to EMS-induced *sh1* seed mutants, which were isolated on the basis of the *sh1* seed-mutant phenotype. All of the isolated *sh1* mutants are defective in *Sh1*-encoded gene product, sucrose synthase (SS1) (Chourey and Schwartz, 1971; Chourey and Nelson, 1979).

Restriction fragment length polymorphism (RFLP) mapping, which was done at the University of Missouri (Columbia) RFLP laboratory (Coe et al., 1995), showed that the *incw2* cDNA clone (Taliercio et al., 1995) mapped to the long arm of chromosome 2, to a position which is indistinguishable from the *mn1* locus (Chourey, personal communication). Another cDNA clone for the CWI enzyme, representing the *incw1* gene

isolated from suspension-cultured cells (Shanker, et al., 1995), mapped to chromosome 5. Of the two soluble forms of invertase (*Ivr1* and *Ivr2*) isolated from maize roots (Xu et al., 1995), the *Ivr1* clone was also mapped to chromosome 2 (short arm), but ~40 map units away from the *mn1* locus (Xu et al, 1996).

There are DNA data showing that the *Mn1* locus cosegregated with the *incw2* gene in the F_2 (or advanced generations) and backcross hybrids. In addition, the *incw2* cDNA clone discriminated various *mn1* mutants into two subclasses. One group of mutants have shown *incw2* transcripts (~ 2.2 kb), such as *mn1-74*, *mn1-84*, and *mn1-89*. The other group have shown undetectable levels of such transcripts , such as *mn1-1*, *mn1-82*, and *mn1-83* (Chourey and Taliercio, personal communication). Moreover, the former group have mutants with either detectable CWI-2 protein (*mn1-89*) or undetectable protein (*mn1-74*, and *mn1-84*) (data not shown). Taken together, these data strongly suggest that the *Mn1* seed locus encodes a CWI. The *incw2* RNA and CWI-2 (or p72) are the gene products of the *Mn1* locus.

Coordinate Control of Cell Wall-Bound and Soluble Forms of Invertase Activities

Based on enzyme-activity assays, both cell wall-bound and soluble forms of invertase appeared to be regulated coordinately at various developmental stages (Figure 3-1) and in various genotypes (Table 3-1). In each case, the decrease or increase of the predominant cell wall-bound form is always associated with the relative alteration of soluble form. This pattern has previously been observed in a limited sample of genotypes (Miller and Chourey, 1992) and has been attributed to a common genetic basis for the two isoenzymes, as is the case in yeast (Carlson and Botstein, 1982). However, recent evidence has shown that these two forms of invertase are encoded by two distinct genes in various plant species examined thus far (Unger et al., 1994; Weil et al., 1994; Greiner

et al., 1995; Roitsch et al., 1995; Weber et al., 1995), including maize (Shanker et al., 1995; Taliercio et al., 1995; Xu et al., 1995).

Additional evidence in support of the coordinate regulation of invertase isoenzymes is the expression of antisense invertase in transgenic tomato (Ohyama et al., 1995). The expression of antisense transgene effectively represses the expression of the cell wall and soluble forms of invertase. Similarly, both forms of invertase in developing kernel grown *in vitro* are also affected under heat stress condition (Cheikh and Jones, 1995).

Such a coordinate regulation of invertases is probably due to the unique gradient of sucrose concentration in the endosperm, the highest at the base and the lowest at the top (Griffith et al., 1987; Doehlert and Kuo, 1990). Although there are higher levels of ^{14}C-sucrose in the *mn1-1* endosperm (Shannon et al., 1993) following a $^{14}CO_2$ pulse into plants of these two genotypes (homozygous *Mn1* and *mn1-1*), a lack of sucrose hydrolysis in *mn1* kernel base (pedicel and basal endosperm transfer cells) must "lock-out" (reducing) sugars from entering developing endosperm and may lead to down-regulation of the intracellular soluble invertase. Consistent with this speculation are our data from maize suspension-cultured cells showing that both cell wall-bound and soluble forms of invertase were coordinately regulated over the time course under sugar starvation (see Chapter 5). In addition, CWI is a sugar-modulated enzyme in suspension-cultured cells of *Chenopodium rubrum* (Roitsch et al,. 1995). Xu et al. (1995) have also reported that two soluble forms of invertase (*Ivr1* and *Ivr2*) displayed differential expression in response to sugar treatment in maize excised root tips. Thus, it appears that invertase is one of the enzymes involved in metabolic control mediated by sugars (for reviews; see Sheen, 1994; Thomas and Rodriguez, 1994; Koch, 1996). The specific details on how

metabolic status of sugars modulates expression of various genes, including invertase, remains to be elucidated.

A Critical Role of Invertase in Stability of Maternal Cells and Sink Strength in Endosperm

In addition to the lack of invertase activity/protein and the loss of seed weight by ~80% of the wild type, the *mn1-1* mutant also shows a withdrawal of the pedicel from the endosperm (i.e., a gap formation) as early as at 10 DAP (Lowe and Nelson, 1946). However, the gap formation can be arrested and seed phenotype is restored to wild type when invertase activity is elevated up to 20% in the endosperm with one copy of *Mn1* allele (i.e., *Mn1mn1-1mn1-1* hybrid obtained by the cross of homozygous *mn1-1* with homozygous *Mn1*) (Miller and Chourey, 1992). The current data presented here extend further the speculation that the stability of placento-chalazal cells in the pedicel is determined by certain levels of invertase in the endosperm. There are at least three features noteworthy in this study concerning stability of maternal cells and sink strength in endosperm.

First, a significantly lower level of invertase activity (~4 to 6% of wild type activity) was sufficient to arrest the gap formation in homozygous *mn1-89* seed mutant and its hybrids with two copies of *mn1-89* allele (i.e., *mn1-89mn1-89mn1-1*, obtained by *mn1-89mn1-89* X *mn1-1mn1-1*). Second, the reciprocal hybrids with one copy of *mn1-89* (i.e., *mn1-89mn1-1mn1-1*, obtained by *mn1-1mn1-1* X *mn1-89mn1-89*), and only ~2% total invertase activity of wild type was distinctly marked by a gap formation, as seen in the *mn1-1* seed mutant; moreover, the seed phenotype of this hybrid was indistinguishable from the *mn1-1* seed mutant. Third, CWIs present in endosperm and pedicel were two isoenzymes, CWI-2 (p72) and CWI-1 (p68), respectively, and are encoded by two distinct genes. The relative enzyme activity in the pedicel was ~ 20% of

wild type when compared to whole kernel (data not shown). The elevated levels of CWI-2 (p72) enzyme in endosperm is associated with a relative increase of CWI-1 (p68) enzyme in the pedicel, as judged by the histochemical staining for invertase activity in the developing kernels with various *Mn1* gene-dose genotypes (Miller and Chourey, 1992). Thus, the correlated changes in anatomical structure and enzyme activity indicate that the invertase-dependent metabolic status of endosperm affected the stability of cells and CWI-1 gene expression in the pedicel.

The interdependence of the pedicel on endosperm, as in the *mn1-1* seed mutant, is believed to be due to a transient accumulation of sucrose and an osmotic imbalance in the pedicel. Consequently, it causes the degeneration of placento-chalazal cells in this region (Miller and Chourey, 1992). This hypothesis is strengthened by the observation of the elevated levels of ^{14}C-sucrose in the pedicel of *mn1-1* relative to *Mn1*, following a $^{14}CO_2$ pulse (Shannon et al., 1993). Perhaps, this is the most direct evidence that CWI plays a crucial role in osmoregulation and/or turgor sensing in sugar transport, as has been suggested previously based mainly on inferential results (von Schaewen et al., 1990; Ramloch-Lorenz et al., 1993; Weber et al., 1995).

Because invertase is spatially and temporally the first enzyme in developing endosperm to metabolize incoming sucrose, it is reasonable that the loss of the invertase activity in the *mn1-1* mutant leads to a significant reduction of seed weight (or sink strength). In fact, these data from the *mn1-89* mutant and its hybrids with *mn1-1* parent demonstrate that the enzyme played a rate-limiting role in sink strength in developing kernel, as evidenced by the increase in seed weights in a *mn1-89* gene-dose-dependent manner (Table 3-2). However, such a rate-limiting role of invertase occurs only when the enzyme activity is below 6% of the normal wild type. Since the *mn1-89* mutant with only 6% activity of wild type showed nearly wild-type seed phenotype and possessed ~80%

wild-type seed weight, it is reasonable that a genotype with slight increase of the enzyme activity, perhaps ~8 or 10% of wild type, will be restored to normal wild-type phenotype. On the biochemical basis, it is likely that kernel with 10% of total invertase activity gains a sufficient level of sugars, among other factors, for normal metabolism of the developing endosperm. However, when invertase activity below this value (10%), sugars or other photoassimilates are limited below the minimum requirements of normal metabolism, causing the altered expression of metabolic and other related genes, such as SPS and SS proteins (see Chapter 6). Finally, such adaptation leads to significant seed phenotype changes. Thus, although a substantial level of invertase activity (~90%) is dispensable, there was a threshold value (~10%), below which the enzyme may play a rate-limiting role in the sink strength of developing kernel and the stability of maternal cells in the pedicel. A large excess of enzyme activity appears to be a common feature with many enzyme systems in diverse plant species as was described previously (Chourey and Nelson, 1979; Quick et al., 1991; Zrenner et al., 1993).

Figure 3-1. Developmental profile of invertase activity and protein in immature kernels.

(A) Specific activity values are shown for crude extracts of homozygous *Mn1* kernels harvested at various developmental stages. The highest level of activity was at 12 DAP for *Mn1* kernels. The *mn1-1* kernels have shown only low to undetectable levels of activity at 12 to 24 DAP. Values represent means of duplicate measurements with standard deviations <4%; the results are reproducible in at least two sets of independent experiments.

(B) SDS immunoblot analysis shows the p72 CWI polypeptide (CWI-2) in kernel extracts from lower (odd numbers) and upper (even numbers) parts of kernels harvested at 12 (lanes 1 and 2), 16 (lanes 3 and 4), 24 (lanes 5 and 6), 28 (lanes 7 and 8), and 32 (lanes 9 and 10) DAP. Each lane represents 15 (lanes 1 and 3) and 60 μg (lanes 2, 4, and 5 to 10) of protein in crude extracts, respectively.

(C) SDS immunoblot analysis shows the CWI polypeptide in extracts from the pedicel (lane 1), whole kernel (12 DAP) without the pedicel (lane 2), and suspension culture cells (lane 3). The amount of protein in crude extracts is as follows: lane 1, 80 μg; lane 2, 40 μg; lane 3, 50 μg. The CWI polypeptide in the pedicel and suspension culture cell extracts is estimated as ~68 kDa (CWI-1), whereas it is ~72 kDa (CWI-2) in endosperm.

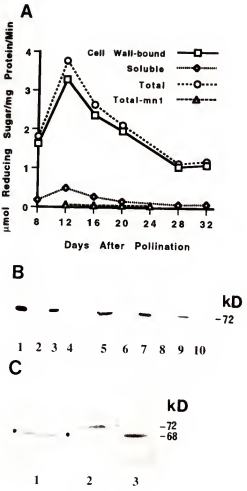

A
µmol Reducing Sugar/mg Protein/Min
Cell Wall-bound
Soluble
Total
Total-mnl
0
1
2
3
4
8 12 16 20 24 28 32
Days After Pollination
B
kD
-72
1 2 3 4 5 6 7 8 9 10
C
kD
-72
-68
1 2 3

Table 3-1. Gene-Dose Relationship with Enzyme Activity in Various Genotypes of Immature Kernels at 12 DAP

	Enzyme Activity[a] (μmol reducing sugar/mg protein/min)					
Genotype	Bound	(%)[b]	Solu.[c]	(%)	Total	(%)
mn1-89 versus *mn1-1*						
Mn1Mn1Mn1	1.752±0.004	(100)	0.225±0.003	(100)	1.980±0.007	(100)
mn1-89mn1-89mn1-89	0.084±0.013	(4.8)	0.033±0.004	(14.7)	0.117±0.017	(5.9)
mn1-89mn1-89mn1-1	0.052±0.007	(3.0)	0.025±0.000	(11.4)	0.077±0.007	(3.9)
mn1-89mn1-1mn1-1	0.025±0.020	(1.4)	0.019±0.010	(8.4)	0.044±0.030	(2.2)
mn1-1mn1-1mn1-1	0.020±0.003	(1.1)	0.008±0.007	(3.6)	0.028±0.010	(1.4)
Mn1 versus *mn1-1*						
Mn1Mn1Mn1	1.516±0.000	(100)	0.276±0.004	(100)	1.792±0.004	(100)
Mn1Mn1mn1-1	1.232±0.008	(81.2)	0.176±0.007	(63.8)	1.408±0.015	(78.6)
Mn1mn1-1mn1-1	0.227±0.008	(15.0)	0.150±0.000	(54.3)	0.377±0.008	(21.0)
mn1-1mn1-1mn1-1	0.022±0.014	(1.4)	0.011±0.014	(4.0)	0.033±0.028	(1.8)
Mn1 versus *mn1-89*						
Mn1Mn1Mn1	1.819±0.007	(100)	0.191±0.004	(100)	2.010±0.011	(100)
Mn1Mn1mn1-89	1.149±0.007	(63.4)	0.171±0.003	(89.5)	1.320±0.010	(65.7)
Mn1mn1-89mn1-89	0.283±0.002	(15.6)	0.114±0.004	(59.7)	0.397±0.006	(19.8)
mn1-89mn1-89mn1-89	0.078±0.000	(4.3)	0.034±0.001	(17.8)	0.112±0.001	(5.6)

[a] Values represent means of duplicate measurements±SD; the results are reproducible in at least two sets of independent experiments, each from different kernel sources.
[b] Percentage values within parentheses are normalized to homozygous *Mn1* genotype.
[c] Solul., soluble.

Figure 3-2. SDS immunoblots showing a positive correlation between gene dose at the *Mn1* locus and the levels of CWI-2 protein in 12-DAP kernels.

(A) Each lane contained 50 μg of protein in crude extracts from whole kernels of *Mn1/Mn1/Mn1* (lane 1), *Mn1/Mn1/mn1-1* (lane 2), *Mn1/mn1-1/mn1-1* (lane 3), and *mn1-1/mn1-1/mn1-1* (lane 4) homozygotes or hybrids.

(B) The same gel as shown in (A), except only the lower third of the kernels was used for preparing the extracts.

(C) Each lane contained 50 μg of protein in crude extracts from the lower third of kernels of *Mn1/Mn1/Mn1* (lane 1), *Mn1/Mn1/mn1-89* (lane 2), *Mn1/mn1-89/mn1-89* (lane 3), and *mn1-89/mn1-89/mn1-89* (lane 4) homozygotes or hybrids.

A faint band representing extremely low levels of CWI-2 protein was detected in homozygous *mn1-89* but not in the *mn1-1* mutant.

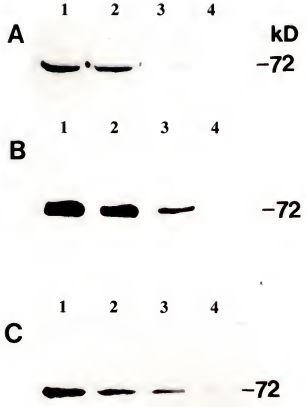

1 2 3 4
A
kD
-72
1 2 3 4
B
-72
1 2 3 4
C
-72

Figure 3-3. Relationship between the copy number of the *mn1-89* allele and the anatomical continuity between the pedicel and endosperm.

Longitudinal sections of 12- to 16-DAP kernels representing the basal endosperm and pedicel are shown. There was no gap between the endosperm and the pedicel in genotypes with three and two copies of the *mn1-89* allele shown in (A) and (B), respectively; however, the reciprocal hybrid with a single copy of the *mn1-89* allele shown in (C) developed a gap similar to that seen in the homozygous *mn1-1* parent shown in (D). Bar in (A) = 65 μm; em, embryo; en, endosperm; g, gap; p, pedicel.

(A) *mn1-89/mn1-89/mn1-89* homozygote.

(B) *mn1-89/mn1-89/mn1-1* hybrid.

(C) *mn1-89/mn1-1/mn1-1* hybrid.

(D) *mn1-1/mn1-1/mn1-1* homozygote.

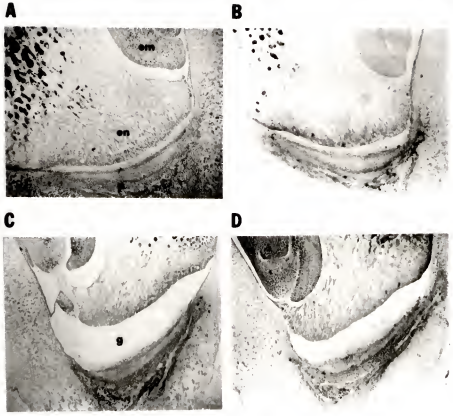

A
B
C
D
em
en
g

Figure 3-4. Variability in seed phenotypes is dependent on the copy number of the *mn1-89* allele.

Seed size and anthocyanin pigment are dependent on the copy number of the *mn1-89* allele. Endosperm genotypes from left to right are *Mn1/Mn1/Mn1*, *mn1-89/mn1-89/mn1-89*, *mn1-89/mn1-89/mn1-1*, and *mn1-89/mn1-1/mn1-1*.

1 2 3 4

Table 3-2. Gene-Dose Relationship of the *mn1-89* Allele with Seed Weight or Sink Strength.

Cross[a]	Phenotype[b]	Segregation Ratio	*mn1-89*[c] allele	Seed Weight(g)[d] Mutant/WT (%)	No.of Kernels[e]
Mn1mn1-89⊗	semi/WT	1/3.14	3	13.75/17.78 (77.3)	76
Mn1mn1-89 X mn1-1mn1-1	semi/WT	1/1.16	2	4.60/6.50 (70.8)	30
Mn1mn1-1 X mn1-89mn1-89	mini/WT	1/1.02	1	6.60/18.12 (43.5)	88
Mn1mn1-1⊗	mini/WT	1/2.60	0	8.78/20.22 (36.4)	100

[a] ⊗ and X represent self- and cross-pollination, respectively.
[b] semi, WT, and mini represent semi-miniature, wild type, and miniature seed phenotypes, respectively.
[c] The values represent the copy number of *mn1-89* allele in mutant seeds.
[d] Values in parentheses represent relative seed weight of semi-miniature (or intermediate) or miniature mutant seeds against their corresponding wild-type seeds on the same ear. The results were reproducible in two sets of independent experiments, each from different kernel sources.
[e] The values represent the number of kernels used for seed-weight measurement in each genotype.

Figure 3-5. Immunolocalization of CWI protein in 12-DAP basal endosperms and pedicels in various genotypes.

The cell wall invertase protein was predominantly localized to wall ingrowths and the apoplastic region in basal endosperm cells in *Mn1* kernels (B), (C), and (D); positive signal was also visible along upper vascular bundles of the pedicel (B) and (C). However, there was no significant signal seen in homozygous *mn1-89* (E) and *mn1-1* (F) seed mutants. Bars = 65 μm in (A); 13 μm in (C); 2.6 μm in (D). The scale in (B) and (E) to (G) is the same as in (A). em, embryo; en, endosperm; g, gap; p, pedicel.

(A) *Mn1/Mn1/Mn1*, treated with preimmune serum.

(B) to (D) *Mn1/Mn1/Mn1*.

(E) *mn1-89/mn1-89/mn1-89*.

(F) *mn1-1/mn1-1/mn1-1*.

(G) *mn2/mn2/mn2*.

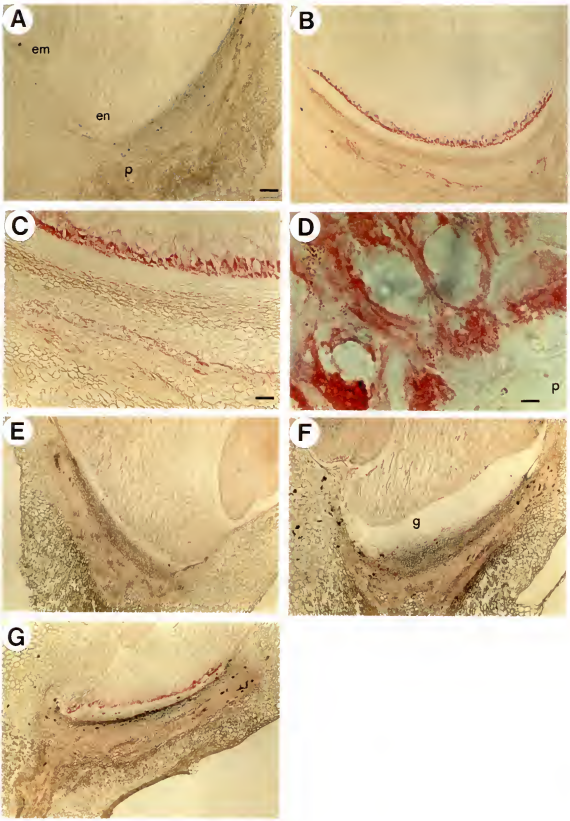

A
em
en
p
B
C
D
p
E
F
g
G

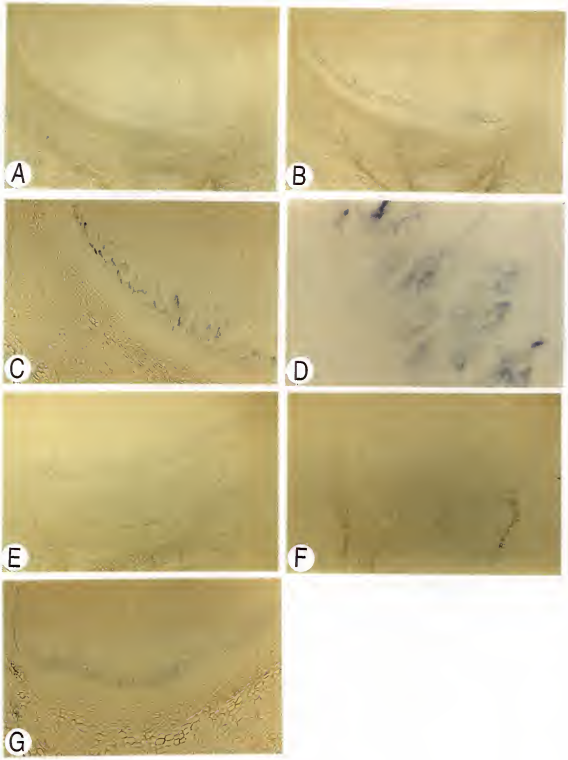
Figure 3-6. *In situ* hybridization of cell wall-bound invertase in developing kernel at 12 DAP.

(A) *In situ* hybridization with digoxigenin-labeled *incw2* sense construct on kernel longitudinal-section of homozygous *Mn1* genotype. (12.5X)

(B), (C), and (D) *In situ* hybridization with digoxigenin-labeled *incw2* anti-sense RNAs on kernel longitudinal-section of homozygous *Mn1* genotype. (12.5X, 62.5X, and 312.5, respectively)

(E), (F), and (G) *In situ* hybridization with *incw2* anti-sense RNAs on kernel longitudinal-sections of homozygous *mn1-89*, *mn1-1*, and *mn2* genotypes, respectively. (31.3X, 12.5X, and 31.3X, respectively)

The *incw2* transcripts were seen in the basal endosperm transfer cells with homozygous *Mn1*, *mn1-89*, or *mn2* genotypes, but low to undetectable in the pedicel. RNA signal was visualized as blue-purple stain under light field.


A
B
C
D
E
F
G

CHAPTER 4
EFFECTS OF SUGARS ON THE *MINIATURE1* SEED MUTANT PHENOTYPE AND SUCROSE-METABOLIZING ENZYMES IN COB IN KERNEL CULTURE

Introduction

The grain yield of maize is primarily dependent on the kernel starch content, which is responsible for ~88% of seed weight (Glover and Mertz, 1987). Sucrose transport to developing endosperm, an active storage tissue, is a critical step in supplying carbon for basic metabolic processes, including starch biosynthesis. To mimic sucrose transport and metabolism in the developing kernel *in planta*, *in-vitro* kernel culture has been utilized in this study. Gengenbach (1977) utilized maize unfertilized kernels attached to pieces of cob to culture *in vitro*, to investigate pollination of unfertilized ovaries. After pollination, the fertilized kernels grow and develop to maturity. This method has been utilized to study sugar transport/utilization (Cobb and Hannah, 1983, 1986, 1988; Hanft and Jones, 1986a, 1986b; Griffith et al., 1987; Schmalstig and Hitz, 1987; Cobb et al., 1988; Felker, 1992), nitrogen metabolism (Cully et al., 1984; Misra and Oaks, 1985) or nitrogen/carbon interaction (Singletary and Below, 1989, 1990), as well as effects of environmental stress, such as temperature (Jones et al., 1981), on kernel development.

Several studies have been done to elucidate sucrose transport from the pedicel into endosperm. One group suggests that sucrose is first cleaved into its components, fructose and glucose in the pedicel by the action of acid invertase, then absorbed into endosperm by basal endosperm cells (Shannon, 1968, 1972; Shannon and Dougherty, 1972; Porter et

al., 1985). The other possibility is that sucrose may enter the endosperm without hydrolysis (Cobb and Hannah, 1986, 1988; Schmalstig and Hitz, 1987; Felker, 1992). Doehlert and Felker (1987) have suggested that both sucrose and its hydrolytic breakdown products might enter simultaneously into the endosperm.

The interruption of (reducing) sugar flux into the basal endosperm cells, for instance, as seen in the invertase-deficient *miniature1* (*mn1*) seed mutant (Miller and Chourey, 1992), causes the ~80% loss of seed weight and is accompanied by the degeneration of placento-chalazal cells, thereafter forming a gap between pedicel and endosperm (Lowe and Nelson, 1946; Miller and Chourey, 1992). Such an anatomical alteration is presumably due to the loss of invertase in this mutant at both pedicel and kernel base regions, causing an osmotic imbalance in this region (Miller and Chourey, 1992). Conceivably, one might speculate that the *mn1* seed phenotype might be restored to near normal or even fully wild-type phenotype when homozygous *mn1* kernels are grown on the reducing sugars fructose or glucose. Such a restoration could be due to the direct entry of reducing sugars from pedicel into endosperm, regardless of the presence or absence of acid invertase.

Normally, maize kernels do not grow and develop *in vitro* without the attachment of cob tissue (Felker, 1992), indicating an important role for this tissue in nutrient uptake and transport. The transport of sucrose taken up by the cob tissue from the medium into the pedicel either as sucrose (Felker, 1992) or as its breakdown products (Hanft and Jones, 1986b) has been suggested. More work needs to be done to better understand the effect of cob tissue on sugar uptake from the medium and subsequent transport into pedicel or endosperm.

In this study, there are two main objectives. First, to test effects of sucrose, fructose, or glucose on *mn1* kernels under *in-vitro* condition, the resulting seed

phenotype, invertase activity, and protein were examined. Second, to investigate possible effects of cob tissue on sugar uptake and transport into the pedicel, sucrose-metabolizing enzymes in cob tissue *in vitro* in comparison to *in planta* were analyzed. The results presented here suggest that the *mn1* seed mutant grown on various sugars retains the mutant phenotype. A possible effect of the sucrose-metabolizing enzymes, sucrose-phosphate synthase (SPS) and sucrose synthase (SS) in cob tissue on sucrose metabolism is discussed.

Plant Materials and Methods

Plant Materials

Homozygous *Mn1Mn1* maize plants used in this study were either Wisconsin 22 (W22) or Pioneer 3165 inbred lines; the *miniature1* (*mn1*) seed mutant was in W22 genetic background. All plants were grown in the greenhouse or field; the temperature was controlled between 28-32°C in the former with a normal diurnal (day/night) pattern. Field-grown plants were normally planted in April, from 1993 to 1996 on the UF campus. Young ears at 3 days after pollination (DAP) were harvested and immediately brought to the laboratory for subsequent kernel culture under aseptic conditions.

Kernel Culture and Growth Conditions

After removing ~4 layers of outer husks, young ears were sterilized with a 95% alcohol-spray and dried for two minutes under the laminar flow hood. Then, inner husks and silks were removed using forceps. In a moist dish, a scalpel was used to longitudinally dissect the ear into two-row pieces with a total of six-attached kernels, each row with 3 kernels. After further severing most of the white pith tissue and

removing 3 kernels from alternating rows of block, final blocks with 3-attached kernels were placed on 100 x 25 mm plastic petri dishes, each plate with 4 blocks, containing various sugar-supplemented media, and incubated in a dark growth chamber at 28°C. The preparation of media was essentially as described by Gengenbach and Green (1975), and is listed in the Appendix. After 14 to 16 days of culture, the blocks were rinsed with distilled water to remove residual medium components and drained on Kimwipes for about two minutes. Subsequently, kernels with pedicels and cob tissues were separated with a scalpel; some of them were immediately fixed in formalin acetic alcohol (FAA) for immunohistochemical analysis as described below and the remainder were frozen by adding liquid N_2, followed by storage at -80°C until use.

Immunohistochemical Localization

Developing kernels or cob tissues *in planta* or *in vitro* at various stages were harvested and immediately fixed in formalin acetic alcohol (FAA), dehydrated through tertiary butyl alcohol (TBA) series, infiltrated in Paraplast plus paraffin (Fisher Scientific), embedded and immunostained essentially following the protocol described by Chen and Chourey (1989) (see Appendix), with the few exceptions noted below. In brief, to ensure the complete removal of tertiary butyl alcohol from over-night infiltrated kernels, they were subjected to three changes of fresh liquid Paraplus, each for 2 to 3 hr, before embedding. Cross or longitudinal sections were cut ~10 to 12 μm in thickness by using a rotary microtome. Paraffin was removed from sections on the slide in xylene and sequentially hydrated to 30% ethanol. After further washing in distilled water and PBS, slides were incubated with primary antibodies raised against cell wall-bound invertase or sucrose synthase, or preimmune serum as a negative control for ~4 hr. Cell wall-bound invertase and sucrose synthase antibodies were raised in rabbit against carrot cell wall-

bound invertase and maize sucrose-synthase SS1 protein, respectively. Cell wall-bound invertase antibody was a gift from Dr. Arnd Sturm (FMI, Basel, Switzerland). Polyclonal sucrose synthase antibodies (Chourey et al., 1991), recognizes both SS1 and SS2 proteins. The slides were then incubated with secondary antibody solution composed of biotinylated anti-mouse anti-rabbit immunoglobulin and streptavidin alkaline phosphatase. Immunolocalized signals of cell wall-bound invertase and sucrose synthase proteins were visualized using New Fuchsia chromogen (Dako Corp., Carpinteria, CA), resulting in a precipitate of fuchsia-colored end product at the site of the antigen.

SDS Immunoblot Analysis

Crude extracts were prepared from frozen kernels or cob tissues at various developmental stages *in planta* or *in vitro*. Some pith tissues from frozen cob tissues were separated automatically from the vascular parts of cob tissues. Samples were first ground into a fine powder by adding a small amount of liquid N_2 into a chilled mortar and pestle, immediately followed by homogenization with extraction buffer in a 1:4 (tissue:buffer) ratio. Extraction buffer for SPS protein contained 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT and 0.1% (w/v) Triton X-100 (Huber and Huber, 1992). The crude-extract preparation for sucrose synthase or invertase is described below (see, Invertase Enzyme Assays). Crude homogenate was centrifuged at 14,000*g* for 1 min in a microfuge at 4°C. One part of the supernatant was removed for 4X SDS buffer treatment to a final volume containing 1X SDS buffer (60 mM Tris, 2% SDS, 10% glycerol, 5% mercaptoethanol), followed by heating in boiling water for 3 min to denature proteins. An aliquot of supernatant was used for protein assay according to Lowry et al. (1951). The denatured protein samples were separated by an SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). For gel blot analyses, proteins were

electroblotted onto nitrocellulose membranes (Schleicher & Schuell) and treated according to the instructions provided with a Du Pont staining kit (New Renaissance). Briefly, the membrane was blocked in 5% nonfat dry milk in 10 mM PBS-T (PBS-Tween 20) for 1 hr. After two washes with PBS-T, each for 5 min, the membrane was incubated with SPS or cell wall-bound invertase polyclonal antibodies or SS1/or SS2 monoclonal antibody (Chourey et al., 1991) for 1 to 2 hr. SPS antibody was a gift from Dr. Toni A. Voelker (Calgene, Inc., Davis, CA). After the first wash for 15 min and four subsequent 5 min washes, the membrane was incubated with the anti-rabbit or anti-mouse secondary antibody (polyclonal and monoclonal, respectively) immunoglobulin conjugated with horseradish phosphatase (Sigma) for 1 hr. After four washes with PBS-T, the membrane was treated with chemiluminescence reagent (Nel-100; Du Pont) for 1 min before developing.

Invertase Enzyme Assays

Frozen kernels or cob tissues were homogenized in extraction buffer in a 1:10 (w/v) ratio by using a chilled mortar and pestle. The extraction buffer used in the isolation of soluble invertase protein contained 50 mM Tris-maleate, pH 7.0, and 1 mM DTT (Doehlert and Felker, 1987). The homogenate was centrifuged at 14,000*g* for 10 min; the supernatant was retained and dialyzed overnight prior to soluble invertase assays. For sucrose synthase immunoblot assays, the dialysis was omitted. The pellet was washed three times by resuspending in extraction buffer and centrifuged at 14,000*g* for 10 min. The subsequent pellet was resuspended in extraction buffer containing 1 M NaCl in a 1:2 (w/v) ratio, shaken on a rotating shaker for ~2 to 3 hr at 3°C, and subsequently centrifuged at 14,000*g* for 10 min. The final supernatant, enriched for cell wall-bound invertase, was dialyzed against extraction buffer without NaCl at 3°C for

overnight and assayed for enzymatic activity, as described previously (Tsai et al., 1970; Miller and Chourey, 1992).

Results

Invertase Activity and Seed Phenotypes of Kernels Cultured *in vitro*

In-vitro cultured kernels of *Mn1* and *mn1* genotypes on sucrose, fructose, or glucose for 14 to 16 days, were examined for invertase activity as well as seed phenotypes. As shown in Table 4-1, there are two noteworthy features. First, the homozygous *Mn1* kernels cultured on sucrose-supplemented medium showed ~20% reduction of total invertase activity when compared to kernels grown *in planta*. The reduction was mainly found in the cell wall-bound form, but not in the soluble form. Similar results were also seen in the kernels cultured on fructose or glucose-supplemented medium. However, the enzyme activity decreased by ~83% when kernels were cultured on sugar-deprived medium; this reduction appeared in both cell wall-bound and soluble forms. Second, in each set of the sugar-supplemented experiments, cell wall-bound invertase was the predominant form in terms of specific enzyme activity. In contrast, the specific activity of the soluble form was at the same low level as the *in-planta* activity and remained relatively constant.

Unlike the *Mn1* kernels, the *mn1* kernels showed a slight increase in total invertase activity (Figure 4-2) under *in-vitro* culture conditions than *in planta*. However, enzyme activity levels in *mn1* kernels were extremely low (~3.5 to 6.7%) on all these sugar treatments, as compared to *Mn1* kernels grown *in planta*. The percentage of total invertase activity in the *mn1* seed mutant *in planta*, as seen in Table 4-2, is slightly higher than that in Table 3-1. That is because the method used for CWI protein extract in this

study is less efficient, as compared to that in Chapter 3. Thus, the value of total invertase activity in *in-planta Mn1* kernels in Table 4-2 is less than that in Table 3-1.

Figure 4-1 represents seed phenotypes of kernels cultured on various sugar-supplemented media for 14 to 16 days. Kernels of homozygous *Mn1* genotype retained normal wild-type seed phenotype regardless of the source of carbon, sucrose, fructose, or glucose. Figures 4-1A is example of kernels grown on sucrose-supplemented medium; the kernel size was similar to, or slightly smaller, than that *in planta* at 14 DAP. Similarly, homozygous *mn1* kernels showed no change in seed phenotype, regardless of sucrose, fructose, or glucose. Figures 4-1B is example of homozygous *mn1* kernels grown on fructose. The same seed phenotype was also seen when *mn1* kernels were cultured on sucrose or glucose (data not shown). Typical features of the *mn1* seed mutant phenotype, such as miniature seed size and papery pericarp, were still maintained in this and other sugar treatments.

To eliminate the effect of maternal tissue (pedicel and cob) genotype on the absorption of nutrients from the medium and subsequent transport into endosperm, we grew the progeny of self-pollinated heterozygous *Mn1mn1* kernels on various sugar-supplemented media. The resulting F_2 hybrid displayed segregation of the miniature phenotype in each case; as shown in Figures 4-1C the *mn1* mutant seeds grown on glucose segregated from the normal wild-type seeds on the same cob along with the *Mn1mn1* genotype. Kernel genotypes were judged based on the striking differences of seed appearance, such as seed size and papery pericarp, as well as invertase activity. The enzyme activity in segregating miniature seeds (data not shown) was similar to the values as shown in Table 4-2, whereas the wild-type seeds had a lower total enzyme activity than that seen in corresponding media in Table 4-1. It ranged from ~20 to 60% of normal wild-type activity *in planta* (data not shown), presumably due to the segregation of the

Mn1 allele. The wild-type kernels used for enzyme assay might have mixed genotypes of one, two, or three copies of the *Mn1* locus. Invertase activity in endosperm (triploid), as described previously (see Chapter 3), revealed a gene-dose relationship with the *Mn1* locus. Three copies have the highest activity; one copy (in this case in wild-type seed) has the least amount. Thus, it clearly indicates that the *mn1* seed mutant phenotype seen in kernel culture is not due to the effect of maternal tissue (pedicel and cob) genotype, because, in this case, wild-type and *miniature1* mutant seeds were grown on the maternal tissue with the same *Mn1mn1* genotype. When kernels were cultured on the medium without sugar, they did not grow and appeared only slightly swollen, translucent, and fragile (Figure 4-1G), primarily due to carbon source deprivation.

Immunohistological Analysis of Cell Wall-Bound Invertase in Kernels Cultured *in vitro*

Longitudinal sections of *in-vitro* cultured kernels were also examined for cellular level localization of CWI protein. Cell wall-bound invertase expression in developing kernel with homozygous *Mn1* genotype *in planta* was restricted to the endosperm transfer cells, one to two cell layers of the lowest endosperm, as well as along the upper parts of the vascular bundles of the pedicel (Figures 4-2A and -2B; also see Figure 3-5). Similar results were obtained when homozygous *Mn1* kernels were cultured on sucrose, fructose, or glucose, for 14 days. Figures 4-2C to 4-2F are longitudinal sections of *Mn1* kernels cultured on sucrose (Figures 4-2C and -2D) or fructose (Figure 4-2F). Cell wall-bound invertase was primarily present in a single cell layer of basal endosperm transfer cells (Figures 4-2C, 4-2D, and 4-2F), rather than two cell layers as seen *in planta*, consistent with the loss of enzyme activity by ~ 20 to 30% in kernels cultured *in vitro* (Table 4-1). Relatively faint signal remained visible in the vascular bundles of the pedicel (Figure 4-2F). No signal was detected in the preimmune-treated control sections (Figure 4-2E), in

sections of *Mn1* kernels grown on sugar-deprived medium (Figure 4-2G), or invertase-deficient *mn1* kernels treated with sucrose, fructose, or glucose. Figure 4-3H shows a longitudinal section of *mn1* kernel grown on fructose-supplemented medium. It is noteworthy that a gap between endosperm and pedicel was seen *in vitro* as well as *in planta*; however, the gap was smaller in the *in-vitro* kernels.

Sucrose-Metabolizing Enzymes in Cob Tissue

Although sucrose-metabolizing enzymes are well studied in developing kernels, very little is known about these enzymes in cob tissue. To investigate whether there are invertase, sucrose synthases (SS1 and SS2), or SPS activity/ or protein, present in the cob tissue of developing ears, we analyzed this tissue obtained from *in-planta* and *in-vitro* kernel culture experiments. As shown in Figure 4-3, sucrose synthases, SS1 and SS2 encoded by *Sh1* and *Sus1* loci, respectively, were abundant in cob tissue (Figure 4-3A, lanes 1 and 2) and to a lesser extent in pith tissue (Figure 4-3A, lane 3) *in planta* at 16 DAP. The *sh1* mutant is a *Sh1*-deletion strain (Burr and Burr, 1981) and, thus, lacks the gene product completely (Chourey, 1981). However, another isoform of sucrose synthase, SS2, was specifically recognized by the SS2 monoclonal antibody (Figure 4-3A, lanes 4 and 5). Similar results were also obtained from cob tissue extracts of this mutant. SS1 protein in cob tissue was undetectable by immunoblot assay (Figure 4-3B, lanes 2, 3, and 4), whereas SS2 was readily detectable. It is noteworthy that the amount of sucrose synthase in cob tissue, as judged by band intensity, is comparable to that in kernel at 16 DAP when equal amounts of protein are loaded (Figure 4-3A, lanes 1 and 5, respectively). Furthermore, the levels of sucrose synthase in the cob tissue of unfertilized ears, at 8, 12 and 20 days after silk emergence still possess a considerable amount of sucrose synthase, when compared to fertilized ear at 8 and 12 DAP (data not shown).

When cobs attached to kernels were grown on sugar-supplemented media, SS1 and SS2 proteins were slightly reduced as seen on immunoblot (Figure 4-4). The diminished levels were more pronounced in homozygous *mn1* genotype than in homozygous *Mn1* (Figures 4-4A and 4-4B, respectively). The removal of sugar from the medium led to a marked decline of both SS1 and SS2 proteins (Figure 4-4A, lane 5 and Figure 4-4B, lane 4). These results suggest that sugars are essential carbon source for normal metabolism *in vitro*.

Extracts from cob tissue were also analyzed for SPS protein. SDS immunoblot assays on cob extracts from developing ears grown *in planta* showed no SPS polypeptide in both *Mn1* and *mn1* genotypes (data not shown). In contrast, the SPS protein was detectable when cob tissues with attached kernels of *mn1* genotype were *in-vitro* cultured on various sugar-supplemented media. As shown in Figure 4-5, nearly the same levels of SPS protein were seen in cob tissues, which were cultured on various sugars (Figure 4-5, lanes 2, 3, and 4). As expected, the sugar-starvation cob tissues had no detectable SPS protein (Figure 4-5, lane 5).

Invertase (cell wall-bound and soluble) enzyme activity was low to undetectable in both *Mn1* and *mn1* cob tissues at 12 DAP *in-planta* or *in-vitro* culture conditions (data not shown). Cell wall-bound invertase protein was also undetectable by immunoblot analysis (data not shown).

Immunohistological Analysis of Sucrose Synthase in Cob Tissue

Sucrose synthase was abundant in cob tissue based on immunoblot assays (Figure 4-4). To determine the spatial location of this enzyme at the cellular level in cob tissue, polyclonal antibody against maize sucrose synthase (SS1) protein was used as the primary antibody in this study. This antibody recognizes both SS1 and SS2 proteins (Chourey et

al., 1991). Figure 4-6 shows cross sections of homozygous *Mn1* cob tissue grown *in vitro*, including the longitudinally paired row arrangement of the embedded pedicel, stained with antibody. Positive signal for SS protein, as evidenced by a faint fuchsia-colored end product, was primarily present in vascular bundle system of both cob and pedicel tissues (Figure 4-6B). There was no significant signal observed in preimmune-treated sections (Figure 4-6A).

The structural components of the cob include parenchyma cells, namely pith with larger cells and few dispersed vascular bundles, and a vascular region mainly involving the connection of the pedicel vascular-bundle system. Interestingly, the SS protein was localized to the phloem companion cells (Figures 4-7B, 4-7D, and 4-7F) which are easily distinguishable from their sister cells, sieve elements, by their distinct cell types. Companion cells and sieve elements are derived from an uneven division of the same progenitor cell; the former differs from the latter in their smaller size, dense cytoplasm and abundance of mitochondria. Hence, they are readily distinguishable from each other in the phloem of vascular bundles (Behnke, 1989). Figure 4-7B reveals the connection point of the vascular bundle between the cob and the embedded pedicel (in longitudinal direction, see Figure 4-6, arrowhead with V + P marker). SS protein was localized to the round- or rod-shaped (along pedicel) companion cells of the vascular bundles. Since parenchyma cells (or pith) of cob tissue possessed a lesser amount of SS, as seen in immunoblots (Figure 4-3, lane 3), it is due to the presence of this enzyme in the vascular bundle system dispersed in this region (Figure 4-6). In contrast, there was no signal observed in preimmune-treated sections (Figures 4-7A, 4-7C, and 4-7E). Overall, similar results were also obtained in cross sections from *in-planta* samples (data not shown).

Discussion

Effect of Sugars on Seed Phenotypes of Kernels Grown *in vitro*

The aim of this study is to test whether the *mn1* seed mutant is restored to near wild-type or fully wild-type phenotype (i.e., phenocopies), when it is cultured on the reducing sugars fructose or glucose. Because fructose and glucose are the products of an invertase-catalyzed reaction, the hexose sugars may directly enter the endosperm cells without the action of invertase in the apoplastic space of the pedicel and basal endosperm transfer cells. Surprisingly, the *mn1* mutant-seed phenotype remained unchanged when homozygous *mn1* kernels were grown on various sugars, including the reducing sugars (Figures 4-1B and 4-1C). Moreover, the mutant-seed phenotype seen *in vitro* was not influenced by the genotype of maternal tissues (pedicel and cob), because *mn1* and normal wild-type kernels in F_2 segregating ears grown on maternal tissue of the same genotype (*Mn1mn1*) (Figures 4-1C), were readily distinguishable based on their seed phenotypes and by the levels of their respective invertase activities. Two maize mutants, *sh1* and *sh2*, defective for SS1 and ADP-glucose pyrophosphorylase, respectively, have shrunken mutant-seed phenotype and a reduced starch content in mature seed due to the reduced levels of precursors for starch biosynthesis in these two mutants. These two mutants also retain their mutant-seed phenotypes after *in-vitro* kernel culture on sugar-supplemented media, including reducing sugars (Cobb and Hannah, 1983,1986).

Felker (1992) has suggested that the uptake of sugars from the medium into cob tissue and subsequent loading into the phloem for further transport into the pedicel is via a symplastic path. We have also observed that acid invertase in cob tissue was low to undetectable *in planta* and *in vitro* at the enzyme activity level and also undetectable by immunoblot analysis (data not shown). Thus, sucrose transport *in vitro* from parenchyma

cells of cob tissue into the vascular bundle system is by way of the symplast without apoplastic hydrolysis catalyzed by cell wall-bound invertase. Cobb and Hannah (1988) reported that the wild-type, *sh1*, and *sh2* kernels grown on glucose-supplemented medium, had 2- to 3-fold higher levels of glucose in endosperm than fructose and sucrose. Similarly, when kernels were grown on fructose-supplemented medium, fructose concentration in kernel was higher than glucose and sucrose. It remains obscure that whether sugars transport as hexoses or sucrose in developing kernels grown *in vitro*.

As shown in Figure 4-5, SPS protein was detectable in the *mn1* cob tissue grown *in vitro*, whereas it was low to undetectable *in planta*. It remains unknown whether SPS protein in the *mn1* cob tissues *in-vitro* culture conditions is enzymatically active or not. In addition, we have not yet examined the levels of SPS activity/protein in the *Mn1* cob tissues grown *in vitro*. More work needs to be done to illustrate the physiological role of SPS protein in the *mn1* cob tissue cultured *in vitro*.

Possible Roles of Sucrose Synthase in Cob Tissue

The physiological role of sucrose synthase in sink tissues has been suggested to provide the precursor UDP-glucose for starch and cell wall biosyntheses in plants based on biochemical, genetic, and *Sh1* promoter activity studies (Chourey and Nelson, 1976; Carpita and Delmer, 1981; Basra and Malik, 1984; Delmer, 1987; Mass et al., 1990; Chourey et al., 1991a; Amor et al., 1995; Carlson and Chourey, 1996). The relative amount of sucrose synthase in cob tissues *in planta* is comparable to that in the developing kernels (Figure 4-3). Interestingly, the amount of sucrose synthase protein, as judged by immunoblot analyses, was not significantly affected in the cob tissue of unfertilized ears, when compared to fertilized ears (data not shown). Although the physiological significance of sucrose synthase in cob tissue remains to be elucidated, it is

believe that, at least to some extent, it may contribute to cellulose biosynthesis and may play a role in sucrose transport. Since this enzyme is typically associated with rapid cell division or regions of cell wall extension (Chourey et al., 1991a; Koch et al., 1992; Nolte and Koch, 1993; Amor et al., 1995), it is reasonable to expect the enzyme to be present in cob tissue that is undergoing rapid growth and development.

The presence of sucrose synthase within companion cells might play a role in supplying a carbon source for energy production, which in turn is utilized for sucrose transport through the vascular system. More recent data consistent with this notion is also proposed by Lerchl et al. (1995). They described a phloem-specific removal of pyrophosphate (PPi) in transgenic tobacco resulting in photoassimilate accumulation in leaves and stunted growth. These results may be explained by a decrease of cytosolic PPi, subsequent reduction of glycolysis/respiration or inhibition of PP_i-dependent sucrose breakdown catalyzed by sucrose synthase and therefore long-distance sucrose transport would be inhibited. Moreover, this phenotype was complemented by the insertion of another transgene, a phloem-specific yeast-derived invertase. The expression of the yeast-derived invertase bypassed the PPi-dependent sucrose breakdown and the transgenic plant was restored to normal wild-type phenotype.

Table 4-1. Acid Invertase Activity of Homozygous *Mn1* Kernels Cultured on Various Sugars for 14 Days

Medium	Enzyme Activity[a] (μmol reducing sugar/mg protein/min) Bound	Soluble	Total	(%)[b]
Planta	1.022±0.004	0.136±0.003	1.158±0.001	(100)
+Suc	0.796±0.004	0.136±0.001	0.922±0.011	(79.7)
+Fru	0.822±0.037	0.113±0.019	0.935±0.018	(80.7)
+Glc	0.638±0.123	0.137±0.008	0.775±0.115	(66.9)
-Sugar	0.154±0.008	0.045±0.013	0.198±0.006	(17.1)

[a] Values are means ± SD of two independent experiments, each experiment with two measurements.
[b] Values within parentheses refer to kernels *in planta*.

Table 4-2. Acid Invertase Activity of Homozygous *mn1* Kernels Cultured on Various Sugars for 14 Days

Medium	Enzyme Activity[a] (μmol reducing sugar/mg protein/min) Bound	Soluble	Total	(%)[b]	(%)[c]
Planta(*Mn1*)	1.048±0.226	0.140±0.007	1.188±0.233		(100)
Planta(*mn1*)	0.029±0.011	0.009±0.002	0.038±0.013	(100)	(3.2)
+Suc	0.032±0.001	0.020±0.006	0.051±0.007	(134)	(4.3)
+Fru	0.023±0.004	0.019±0.004	0.042±0.009	(111)	(3.5)
+Glc	0.033±0.000	0.018±0.000	0.051±0.000	(134)	(4.3)
-Sugar	0.042±0.004	0.038±0.001	0.080±0.003	(211)	(6.7)

[a] Values are means ± SD of two independent experiments, each experiment with two measurements.
[b] Values within parentheses refer to *in-planta mn1* kernels.
[c] Values within parentheses refer to *in-planta Mn1* kernels.

Figure 4-1. Seed phenotypes of kernels cultured on various sugars for 14 days.

(A) Homozygous *Mn1* kernels cultured on sucrose-supplemented medium retained the wild-type seed phenotype.

(B) Homozygous *mn1* kernels cultured on fructose-supplemented medium retained the seed mutant phenotype.

(C) Self-pollinated heterozygous *Mn1mn1* kernels grown on glucose-supplemented medium showed segregation, the *mn1* seed mutants segregating among normal wild-type seeds on the same cob blocks.

(D) Homozygous *Mn1* kernels grown on sugar-deprived medium displayed no growth.

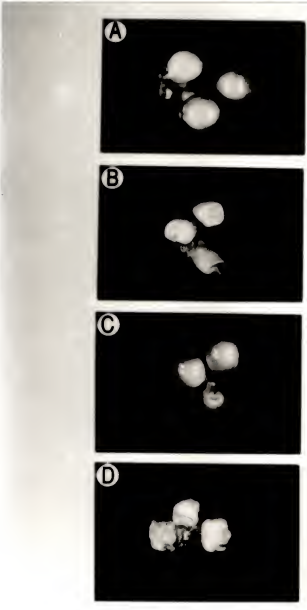

A
B
C
D

Figure 4-2. Immunolocalization of cell wall-bound invertase (CWI) in kernels cultured on various sugar-supplemented media for 14 days.

(A), (B) CWI antibody-treated section of homozygous *Mn1* kernel *in planta*. (B) is a close-up view of section in (A). The magnifications in (A) and (B) are 12.5X and 62.5X, respectively.

(C), (D) CWI antibody-treated section of homozygous *Mn1* kernel grown on sucrose-supplemented medium. (D) is a close-up view of section in (C). The magnifications in (C) and (D) are 12.5X and 62.5X, respectively.

(E) Preimmune-treated longitudinal section of homozygous *Mn1* kernel cultured on sucrose-supplemented medium. This section is from the same kernel as in (C). (12.5X)

(F) CWI antibody-treated section of homozygous *Mn1* kernel cultured on fructose-supplemented medium. (12.5X)

(G) CWI antibody-treated section of homozygous *Mn1* kernel cultured on sugar-deprived medium. (12.5X)

(H) CWI antibody-treated section of homozygous *mn1* cultured on fructose-supplemented medium. (12.5X)

CWI signal was visible only in the sections of *Mn1* kernels *in planta* or grown on sugar-supplemented media (C), (D), and (F). It is noteworthy that CWI in section of *mn1* kernel remained undetectable and kernel still had a gap between endosperm and pedicel (H).

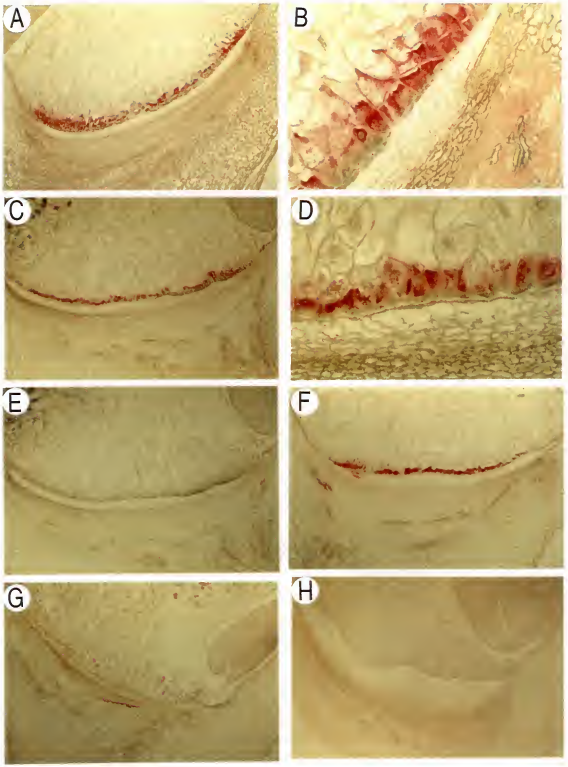
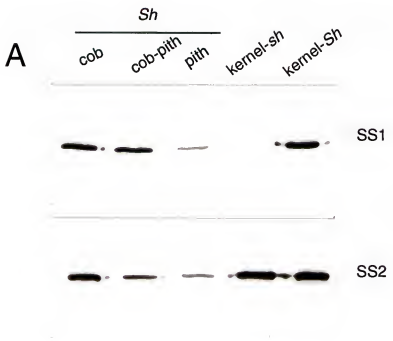

A
B
C
D
E
F
G
H

Figure 4-3. SDS immunoblot analyses of sucrose synthase in cob tissue *in planta*.

(A) Crude protein extracts were prepared from the samples as follows:
Lane 1: cob tissue.
Lane 2: cob tissue without pith.
Lane 3: pith.
Lane 4: *sh1*-deletion mutant kernel at 12 DAP (as a negative control).
Lane 5: *Sh1Sh1* inbred line kernel at 16 DAP.
Samples in lanes 1 to 3, and 5 are from the same ear. Each lane contained 10 μg crude protein extract.

(B) Crude protein extracts were prepared from cob and kernel of *sh1*-deletion mutant at 12 DAP. Wild-type kernel was used as a positive control.
Lane 1: *sh1*-deletion kernel; 2 μg for SS1; 10 μg for SS2.
Lane 2: *sh1*-deletion cob, 10 μg for both SS1 and SS2.
Lane 3: *sh1*-deletion cob, 5 μg for both SS1 and SS2.
Lane 4: *sh1*-deletion cob, 2 μg for both SS1 and SS2.
Lane 5: wild-type (*Sh1*) kernel, 2 μg for SS1; 10 μg for SS2.

SS1 and SS2 monoclonal antibodies were used in this assay. In general, wild-type cob, pith, or kernel contained both SS1 and SS2, while *sh1*-deletion mutant lost SS1 in both kernel and cob tissues.


A
Sh
cob
cob-pith
pith
kernel-sh
kernel-Sh
SS1
SS2

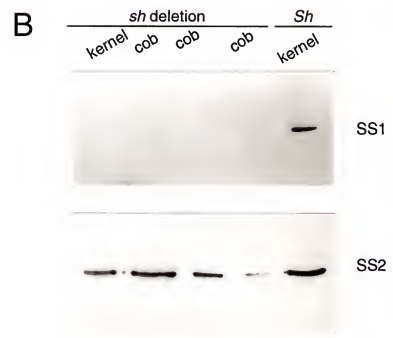

B
sh deletion
Sh
kernel
cob
cob
cob
kernel
SS1
SS2

Figure 4-4. SDS immunoblots showing a comparison of sucrose synthase in homozygous *Mn1* or *mn1* cob tissues grown *in vitro*.

Crude protein extracts were prepared from homozygous (A) *Mn1* and (B) *mn1-1* cob tissues grown on various sugar-supplied media for 14 days. The cob tissues were separated from the attached kernels prior to grinding. Each lane contained 10 μg crude extract. Extract obtained from *in-planta Mn1* kernel at 12 DAP was used as a reference in lane 1 (A). *In-planta Mn1* cobs and kernels at 16 DAP were used as references in lanes 5 and 6 (B).

In general, the amounts of SS1 and SS2 were reduced under *in-vitro* growth conditions as compared *in planta*, in both *Mn1* and *mn1* genotypes.

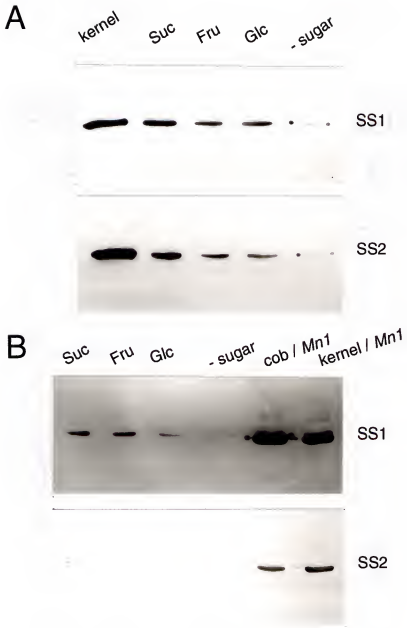

A
kernel
Suc
Fru
Glc
- sugar
SS1
SS2
B
Suc
Fru
Glc
- sugar
cob / Mn1
kernel / Mn1
SS1
SS2

Figure 4-5. SDS immunoblot showing the induction of SPS protein in cob tissue cultured *in vitro*.

Homozygous *mn1* cob tissues with the attached kernels were grown on various sugar-supplemented media for 14 days. Cob tissues were separated from kernels before the preparation of crude extracts. Extract obtained from *in-planta Mn1* kernels at 16 DAP was used as a reference (lane 1) since SPS in cob tissue *in planta* is low to undetectable. Each lane contained 100 μg crude extract.

The levels of SPS protein in cob tissue under *in-vitro* culture conditions were slightly elevated, as no SPS protein was seen in extracts prepared form cobs grown *in planta*.

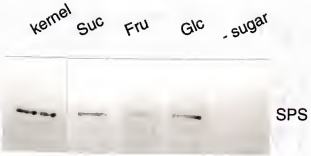

kernel
Suc
Fru
Glc
- sugar
SPS

Figure 4-6. Immunohistological analysis of sucrose synthase in cob tissue grown *in vitro*.

Cross sections were made from the cob tissue cultured on glucose-containing medium for 14 days. Cob tissues were separated from kernels prior to fixation. Sucrose synthase protein was localized to the phloem portion of the vascular bundles in cob as well as pedicel tissues.

(A) Preimmune-treated control. (12.5X).

(B) Sucrose synthase polyclonal antibody-treated section (12.5X); v, vascular bundle (in the cob); p, vascular bundle (in the pedicel).

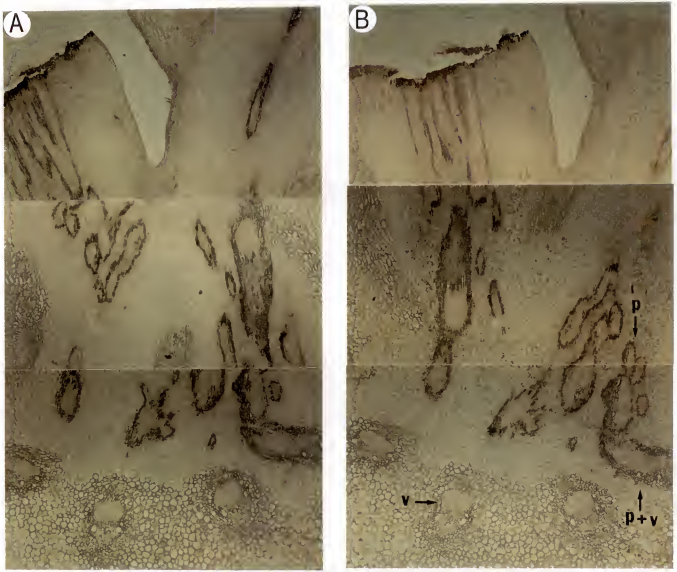
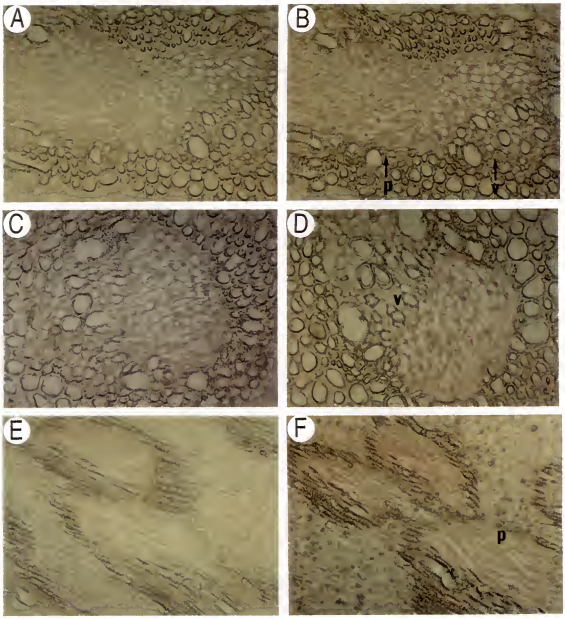

A
B
p
v
p+v

Figure 4-7. Immunohistological analysis of sucrose synthase in cob tissue grown *in vitro*.

This figure is a higher magnification (62.5X) at the arrowhead-marked regions (V + P, V, and P, respectively) of Figure 4-6. Sucrose synthase protein was restricted to the companion cells of the phloem regions in both cob and pedicel tissues, as evidenced by the fuchsia-colored end product (faint red color) at the site of antigen.

(A), (C), and (E) Preimmune-treated controls; v, vascular bundle (in the cob); p, vascular bundle (in the pedicel).

(B), (D), and (F) Sucrose synthase polyclonal antibody-treated sections.


A
B
p
v
C
D
v
E
F
p

CHAPTER 5
SUGAR-MODULATED EXPRESSION OF ACID INVERTASE AND SUCROSE SYNTHASE IN MAIZE SUSPENSION-CULTURED CELLS

Introduction

Sucrose-metabolizing enzymes, sucrose synthase (SS), sucrose-phosphate synthase (SPS), and/or invertase, are normally present together in the same sink tissues (Geigenberger and Stitt, 1991) for metabolizing sucrose and determining carbon partitioning in these tissues. However, the spatial and temporal expression of invertase (Weber et al., 1995; Xu et al., 1996), SS (Chen and Chourey, 1989; Heinlein and Starlinger, 1989; Rowland et al., 1989; Koch et al., 1992), and SPS (see Chapter 6) in the same tissues are differential. In addition, these enzymes are modulated by sugars, among other factors, in various plant tissues or suspension-cultured cells (for invertase: Roitsch et al., 1995; Xu et al., 1996; for sucrose synthase: Mass et al., 1990; Koch et al., 1992; for SPS: Hesse et al., 1995; also see Chapter 4; for reviews: Koch, 1996). Nevertheless, there is still no report that whether these enzymes also express in suspension-cultured (i.e., undifferentiated) cells of maize or other plant species.

Sugars also act to repress photosynthetic enzymes and chlorophyll synthesis in maize protoplasts of mesophyll cells (Sheen, 1990, 1994), chlorophyll a/b binding protein, *rbcS* in spinach leaf (Krapp et al., 1993), and α-amylase in rice suspension-cultured cells (Yu et al., 1991).

Recently, invertase has been extensively studied in a wide variety of plant species *in vivo* and *in vitro*. It is a sugar-inducible enzyme and shows a differential expression of soluble and cell wall-bound forms in cell suspension culture of *Chenopodium rubrum* (Roitsch et al., 1995). Two soluble invertase genes (*Ivr1* and *Ivr2*) also reveal constrasting response in maize excised root tips under sugar treatment or starvation (Xu et al., 1996). Similarly, the contrasting responses of two isoenzyme genes to exogenous sugars (sugar-enhanced or -repressed) or anaerobic conditions were observed in two SS genes (*Sh1* and *Sus1*) in maize (Rowland et al., 1989; Koch et al., 1992) and α-amylases in rice suspension-cultured cells (Yu et al., 1991). Interestingly, the total gene products or enzyme activities display no significant net change under these stresses (Rowland et al., 1989; Koch et al., 1992). To date, very little is known concerning such contrasting responses of isozyme genes to sugars in two cell wall-bound invertase (CWI) isoforms of the same class genes, *incw1* and *incw2*, in maize suspension-cultured cells. Moreover, the coordinate regulation of soluble and cell wall-bound forms of invertase activities occurred at various developmental stages and in various genotypes (see Chapter 3). It is still obscure that whether such a coordinate control of two invertase activities from different classes is also present in maize suspension-cultured cells.

It is believed that suspension-cultured cell system is a good model to monitor gene expression under defined conditions. The major objectives of this study are to test whether the cell wall-bound and soluble forms of invertase also show coordinate control, as seen in developing endosperm, and whether the expression of invertase is regulated by specific sugars, sucrose, fructose, or glucose. Sugar-modulated expression of the other sucrose-metabolizing enzymes (SS and SPS) were also analyzed. Overall, the data presented here suggest that the coordinate regulation of cell wall-bound (*incw1*) and soluble forms of invertase activities also existed in maize suspension-cultured cells under

sugar stress. Contrasting responses of two SS isozyme genes (*Sh1* and *Sus1*) to sugar stress were also observed.

Plant Materials and Methods

Induction and Maintenance of Callus and Suspension-Cultured Cells

For callus induction and maintenance, homozygous *Mn1* wild-type and *miniature1* (*mn1-1*) mutant plants were grown in the field or green house. After selfing or sibling of homozygous *Mn1* or *mn1-1*, or crossing *mn1-1* (female) with *Mn1* (male) for 7 to 10 days after pollination (DAP), young ears were detached and immediately brought to laboratory for further aseptic manipulation. After removing ~4 layers of outer husks, young ears were sterilized by a 95% alcohol spray and dried for ~ 2 min on the larminar flow hood. Then, inner husks and silks were removed using forceps. In a glass petri dish with wet filter papers, immature kernels were excised from the ear, and immature embryo, ~1 to 2 mm, were picked up using a fine-point forceps and placed on plastic petri dish (100 x 10 mm) with agar medium supplemented with modified MS medium (Murashige and Skoog, 1962) (see Appendix) and 2 mg/l 2,4-D. In general, calli were induced after one-month culture; calli with friable and compact properties were maintained by transferring into 125 ml flask with 50 ml liquid medium containing the same components as mentioned above, except no agar added. Subculture was performed weekly by replacing ~25 ml old medium with the same amount of fresh one. After every 2 to 3-month culture, the smaller calli were collected for maintenance and the larger ones were harvested and frozen by adding liquid N_2 followed by -80°C storage until use. Since these cell lines did not suspend in the medium even after 2- or 3-year culture, they were designated as callus to distinguish from suspension-cultured cells as described below.

A suspension-cultured cell line used in this study has been maintained for many years in the laboratory of Prem S Chourey. It was obtained by squashing a small piece of callus into liquid medium; the callus was induced from the mesocotyl tissue of germinated seeds of 'Black Mexican Sweet' line as mentioned previously by Chourey and Zurawski (1981). The components of the medium were the same as above, with an exception that 4 mg/l 2,4-D was used. This cell line was subcultured once a week by transferring ~0.4g fresh weight of cells into 125 ml flask with 50 ml fresh medium. In each sugar-treated or depleted experiment, suspension-cultured cells were pooled together followed by three washes with autoclaved sugar-free medium; subsequently, they were equally dispersed into 50 ml filter-sterilized fresh medium with/or without sugar addition, generally each flask with ~ 0.8g fresh weight of cells. Cultured cells were then harvested at various growth stages and stored in -80°C until use.

Invertase Activity Assays

Crude protein extracts were prepared from frozen suspension-cultured cells or calli in a 1:10 (w/v) ratio of extraction buffer by using a chilled mortar and pestle. The extraction buffer used in the isolation of soluble invertase protein contained 50 mM Tri-maleate, pH 7.0, and 1 mM DTT (Doehlert and Felker, 1987). The homogenate was centrifuged at 14,000*g* for 10 min; the supernatant was removed for soluble invertase assays following overnight dialysis against extract buffer. The pellet was washed three times by resuspending in extraction buffer and centrifuged at 14,000*g* for 10 min. The subsequent pellet was resuspended in extraction buffer containing 1 M NaCl in a 1:2 (w/v) ratio, shaken on a rotating shaker for 2 to 3 hr at 3°C, and subsequently centrifuged at 14,000*g* for 10 min. The final supernatant enriched for CWI, was dialyzed against

extraction buffer without NaCl at 3°C for overnight and assayed for enzymatic activity, as described previously (Tsai et al., 1970; Miller and Chourey, 1992).

SDS Immunoblot Analysis

Parts of crude extracts, either from undialyzed sample for soluble form or dialyzed sample for cell wall-bound form of invertase as described above, were treated with 4x SDS treatment buffer to a final volume with 1x SDS: 60 mM Tris, 2% SDS, 10% glycerol, and 5% mercaptoethanol, followed by heating on boiling water for 3 min.

Denatured protein samples were separated on an SDS-polyacrylamide gel, according to Laemmli (1970). For immunoblot analysis, proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) and treated according to the instructions provided with a Du Pont staining kit (New Renaissance). Briefly, the membrane was blocked in 5% nonfat dry milk in 10 mM PBS-T (PBS-Tween 20) for 1 hr. After two 5 min washes with PBS-T, the membrane was treated for 1 to 2 hr in appropriately diluted solutions of primary antibodies. Polyclonal antibodies raised against carrot cell wall-bound invertase and maize SPS protein, were kind gifts from Dr. A. Sturm (FMI, Basel, Switzerland) and T.A. Voelker (Calgene, Inc., Davis, CA), respectively. Monoclonal antibodies against maize sucrose synthases, SS1 or SS2, have been isolated and analyzed by Chourey et al. (1991b). After the first wash for 15 min and four washes, each for 5 min, the membrane was treated with the secondary antibodies anti-rabbit or anti-mouse (for polyclonal or monoclonal primary antibodies, respectively) immunoglobulin conjugated with horseradish phosphatase (Sigma) for 1 hr. After four washes, the membrane was treated with chemiluminescence reagent (Nel-100; Du Pont) for 1 min before developing.

RNA Blot Analyses

Total RNA was isolated from frozen calli or suspension-cultured cells as described previously (Wadsworth et al., 1988). The RNA was glyoxylated and size-fractionated on a 1.2 or 1.5% agarose gel electrophoresis. Subsequently, RNA was transferred onto a Nytran membrane (Schleicher & Schuell) followed by prehybridization for at least 2 hr in 50 mM Pipes (pH 6.5), 100 mM NaCl, 50 mM sodium phosphate (pH 6.5), 1 mM EDTA, and 5% SDS. The blots were then hybridized in the same solution with 3 x 10^6 cpm/ml ^{32}P-labeled probe overnight at 65°C. Probes used in this study were prepared from cDNA clones corresponding *incw1* (Shanker et al., 1995), *incw2* (Taliercio et al., 1995), *Sh1*, *Sus1* (Gupta et al., 1988), and *Sps* genes (Worrell et al., 1991). Blots were rinsed two times, each for 45 min, in 6x SSC (1x SSC: 0.15 M NaCl, 0.015 M sodium citrate), 5 mM EDTA (pH 8.0), 5 mM sodium phosphate (pH 6.5), 5% SDS and two times, each for 30 min, in 0.2x SSC, 5 mM EDTA (pH 8.0), 5 mM sodium phosphate (pH 6.5), 1% SDS. The blots were exposed to X-ray film at -80°C. The relative band intensities on X-ray films were determined by computing densitometer, model 300A (Molecular Dynamic, Inc., Sunnyvale, CA).

Results

Tissue- or Cell-Specific Expression of Two Isozyme Genes of Cell Wall-Bound Invertase, *incw1* and *incw2*, in Various Plant Tissues, Callus or Suspension-Cultured Cells of Maize

Two maize CWI clones, representing the *incw1* and *incw2* genes, have been isolated and characterized previously (Shanker et al., 1995; Taliercio et al., 1995). RNA blots based on total RNA from various samples were hybridized with these two cDNA clones. As shown previously (Taliercio et al., 1995), the *incw1* RNA was abundant in the suspension-cultured cells (Figure 5-1A, lanes 1, 8, and 9). The hybridization was also

seen in samples extracted from calli induced from immature embryos (diploid) with various *Mn1* gene-dose genotypes (Figure 5-1A, lanes 2 to 4) and root tips with homozygous *Mn1* or *mn1-1* genotype (Figure 5-1A, lanes 10 and 11). A reduced level of hybridization was seen on the RNAs extracted from young shoots (Figure 5-1A, lane 12) and lower part of kernel (Figure 5-1A, lane 5), but not in the upper part of kernel or whole kernel with *mn1-1* or *Mn1* genotype (Figure 5-1A, lanes 6, 7 and 13). Similarly, when the same RNA blot (Figure 5-1A, from lanes 9 to 13) was rehybridized with the probe made from *incw2* cDNA clone, which was isolated from kernel base, *incw2* hybridized to the kernel transcripts (Figure 5-1A, lane 13) and weakly hybridized to transcripts of suspension-cultured cells (Figure 5-1A, lane 9). The transcripts of suspension-cultured cells was abundant in *incw1* form. These data confirmed previous observation that the *incw2* transcripts were restricted to the lower part of endosperm (see Chapter 3). Overall, *incw2* transcripts (or *Mn1* seed locus transcripts) were exclusively present in the kernel base, whereas *incw1* transcripts were primarily found in root tips, young shoots, suspension-cultured cells or callus.

Gene expression of CWI was also analyzed at the protein level. The SDS immunoblot in Figure 5-1B shows that a larger polypeptide subunit (p72 or CWI-2) was only seen in the kernel samples (Figure 5-1B, lane 4); in contrast, the smaller polypeptide subunit (p68 or CWI-1) was present in calli with various genotypes (Figure 5-1B, lanes 1 to 3), suspension-cultured cells (Figure 5-1B, lane 5), root tips with homozygous *Mn1* or *mn1-1* genotype (data not shown), and the pedicels (see Chapter 3, Figure 3-1C).

According to these and previous data (see Chapter 3), the *incw2* transcript and p72 polypeptide were encoded by the *Mn1* gene and were seen exclusively in the lower part of developing endosperm. In contrast, the *incw1* transcript and p68 protein were found in various plant tissues (except in kernel), including suspension-cultured cells, and callus.

Thus, unless stated otherwise, the *incw1* clone was used as a probe to determine the expression of CWI gene in suspension-cultured cells as described below.

Regulation of CWI Gene Expression in Response to Sucrose Supplement or Depletion in Suspension-Cultured Cells

The RNA blot in Figure 5-2B demonstrates that the steady-state levels of *incw1* transcripts were abundant during one-week culture period in a medium supplemented with sucrose (Figure 5-2B, lanes 1 to 6). However, under sucrose-depleted growth conditions for 6, 12, 24 and 48 hr, the steady-state levels of *incw1* transcripts in cultured cells were significantly reduced, by ~50% (Table 5-1), after 12 or 24 hr culture (Figure 5-2A, lanes 3 and 4), as compared to the normal sucrose-supplemented medium. Interestingly, a slightly larger *incw1* CWI transcript was detectable in the total RNAs from cells grown on sugar-depleted or mannitol-supplemented medium for 48 hr (Figure 5-2A, lanes 5 and 6). To confirm whether the larger *incw1* transcript was favored in the absence of sugars, replenishment of 48 hr-starved cells with fresh sucrose-supplemented medium was performed. As shown in Figure 5-2C, the larger transcript (Figure 5-2C, lane 2) disappeared after the starved cells were grown on sucrose-supplemented medium for 12 hr (Figure 5-2C, lane 3); the smaller transcript, normally abundant in sucrose-supplemented medium, was readily detectable after 24 hr culture (Figure 5-2C, lanes 4 to 6). Thus, it is clear that the larger transcript was repressed in the presence of sucrose, but induced in the absence of sugars. No detectable quantities of RNA was isolated from sucrose-depletion for a longer duration, up to 72 hr. Suspension-cultured cells died after growth on sucrose-depleted medium for 5 days, accompanied with a brown-colored medium.

Expression of the CWI gene was also analyzed at the protein level by SDS immunoblot and enzymatic activity assay. As shown in Table 5-2, when suspension-

cultured cells were grown on sucrose-supplemented media for 12, 24, or 72 hr, there were no significant changes in invertase specific activities at both cell wall-bound and soluble forms. In contrast, the sugar-starved cells led to dramatic reductions of total invertase activities in a time-dependent manner. It is noteworthy that both cell wall-bound and soluble forms of invertase activities declined in a coordinate fashion; that is, the decrease of CWI activity is corresponded to the reduced levels in soluble form. The cells starved for 12, 24, and 72 hr possessed total activity approximately 64.2, 28.1, and 16.8%, respectively.

Consistent with the enzyme activity data (Table 5-2), the cells grown on sucrose-depleted medium for 12 hr showed that the CWI-1 protein (p68) was reduced in a time-dependent manner (Figure 5-3A, lanes 6 to 8). The CWI-1 polypeptide was nearly undetectable after the cells were starved for 48 hr. The decline of invertase activity was not due to osmotic effects because similar reduced levels of invertase activity were also observed in cells cultured in the mannitol-supplemented medium with equivalent concentration of sucrose (data not shown).

Regulation of *Sh1* and *Sus1* Gene Expression in Response to Sucrose Supplement or Depletion in Suspension-Cultured Cells

As for sucrose synthase genes, *Sh1* and *Sus1*, relatively constant levels of *Sh1* transcripts were seen in sucrose-supplemented growth conditions (Figure 5-2B, lanes 1 to 6). In contrast, the *Sus1* transcripts were initially in lower steady-state levels during the first 12 hr culture (Figure 5-2B, lanes 1 and 2), but a gradual increase was seen thereafter (Figure 5-2B, lanes 3 to 6). In addition, the expression of *Sh1* and *Sus1* transcripts was contrasting in response to sucrose depletion. The steady-state levels of *Sh1* transcripts in cells grown in sucrose-depleted medium for 6 hr were initially increased by ~2-fold (Figure 5-2A, lane 2), based on the values of relative band intensities (Table 5-1), with a

decline thereafter (Figure 5-2A, lanes 3 to 5). However, the *Sus1* transcripts were sharply reduced, by a factor of 4-fold after 6 hr culture, and 11-fold after 12 hr culture (Figure 5-2A, lanes 2 and 3, respectively; Table 5-1). Thereafter, the reduction was relatively constant at 24 and 48 hr time points (Figure 5-2A, lanes 4 and 5). The sucrose synthase transcripts (*Sh1* and *Sus1*) in the mannitol-fed cells also showed a reduction, but to a less extent (Figure 5-2A, lane 6; Table 5-1), as compared to sugar-starved cells.

The suspension-cultured cells grown in sucrose-supplemented medium maintained relatively constant levels of sucrose synthase proteins over the one-week culture period (Figure 5-3B). However, SDS immunoblots in Figure 5-3 showed that the SS1 polypeptide was elevated within the first 6 hr under sugar starvation (Figure 5-3A, lanes 2 to 4), then remained constant until 24 hr (Figure 5-3A, lanes 5 to 7). Thereafter the SS1 polypeptide showed a slight reduction at 48 hr (Figure 5-3A, lane 8). In contrast, the SS2 protein showed no significant change within 9 hr sugar starvation (Figure 5-4A, lanes 2 to 5), followed by a slight reduction after 12 hr culture (Figure 5-3A, lanes 6 to 8).

Response of Invertase and Sucrose Synthase to Various Sugars and Sucrose Concentrations in Suspension-Cultured Cells

We have also tested effects of fructose or glucose as a carbon source on expression of sucrose-metabolizing enzymes in cultured cells. SDS immunoblots in Figure 5-4 showed that there were no significant change in invertase and sucrose synthases (SS1 and SS2) in each set of experiments, when cells were grown on sucrose, fructose, or glucose for 24 or 72 hr period. Similar results were also obtained when cells were grown on media with 2, 4, or 6% sucrose concentration for 24, 48, or 72 hr (data not shown).

Discussion

Coordinate Regulation of Cell Wall-Bound and Soluble Forms of Invertase Activities

Both cell wall-bound and soluble forms of invertase activities were regulated coordinately in developing endosperm (triploid) (see Chapter 3). The total enzyme activities of the cell wall-bound and soluble forms of invertase were ~90% and 10%, respectively, throughout kernel development (see Chapter 3, Figure 3-1). Moreover, these two forms of invertase were also coordinately regulated among various genotypes dependent on the *Mn1* gene dosage (see Chapter 3, Table 3-1).

Similarly, such a coordinated regulation of cell wall-bound and soluble forms of invertase activities were also seen in maize suspension-cultured cells. These two forms of invertase revealed a coordinate reduction of enzyme activities under sugar-depletion stress (Table 5-2); moreover, the decline of CWI activities was in a good agreement with its corresponding reduced protein levels (CWI-1 or p68) (Figure 5-3A). Hence, there are at least two paired sets of cell wall-bound and soluble forms of invertase in maize occurring in a co-regulated manner. One set is unique in developing endosperm, which is developmentally and genetically regulated. The other is present in suspension-cultured cells under sugar stress. Also, the invertase genes, at least in CWIs, in endosperm and cultured cells are encoded by separate genes.

The possible basis of coordinated regulation of cell wall and soluble forms of invertase activities in maize suspension-cultured cells is probably the withdrawal of sucrose from the medium, which might cause the loss of metabolic stimuli to trigger a signal pathway specific for CWI biosynthesis. Consequently, the impaired CWI expression will be accompanied by the block of hexose influx from the medium into the cultured cells, leading to the utilization of intracellular storage reserves (sucrose)

catalyzed by soluble invertase. Subsequently, the soluble invertase is down-regulated after the sugar pool is depleted. It is likely that a similar mechanism, among other factors, is the basis for coordinate control of the lack of CWI at the kernel base of the *mn1-1* seed mutant, leading to the block of photoassimilate sugar flux from the pedicel into the endosperm and the down-regulation of intracellular soluble form of invertase, as described previously (see Chapter 3).

Co-regulated gene expression also occurs in the genes encoding enzymatic proteins, which catalyze coupled reactions or unrelated reactions in distinct metabolic pathways. For instance, nitrate and nitrite reductases (NR and NiR, respectively), catalyzing the coupled reactions of nitrate into ammonia, are co-regulated under nitrate and light treatments (Faure et al., 1991). Genetic lesion of starch mutants, *shrunken2* (*sh2*) and *brittle2* (*bt2*), leads to the elevated transcripts of the mutants, also accompanied by the increase of zein transcripts (Giroux et al., 1994), despite the reduction of both starch and storage proteins in these mutants (Barbosa and Glover, 1978; Tsai et al., 1978). Thus, it seems to be a common feature for plants to adjust metabolic pathways through coordinate regulation of gene expression under environmental stimuli or genetic lesions.

In maize suspension-cultured cells, the steady-state *incw1* transcripts and CWI-1 protein were relatively constant throughout one-week culture period under sucrose treatment. Under sugar-depleted growth conditions, *incw1* transcripts and CWI-1 protein were significantly reduced after 12 hr. Both CWI-1 protein and cell-wall bound invertase activity were decreased in a near time-dependent manner, confirming that invertase gene expression in tissues or cultured cells can be modulated by sugars. However, sugar-modulated expression of invertase was independent to the carbohydrate sources. Near equal amount of CWI protein was detected when cultured cells were grown in sucrose, fructose, or glucose-supplemented medium (Figure 5-4). Similarly, CWI transcripts in

carrot (Sturm and Chrispeels, 1990) and tobacco crown gall cells (Weil and Rausch, 1990), or CWI activity in tobacco (Ricardo et al., 1972) and sycamore (Copping and Street, 1972) show no significant changes, when grown in various sugars.

Interestingly, a new *incw1* transcript was seen in 48 hr starved or 48 hr mannitol-fed cells; but these cell extracts had undetectable levels of enzyme activity (Table 5-2) and protein (Figure 5-3A). It is clear that translation of this larger transcript was influenced under such strict starvation stress. A molecular basis of the larger transcript remains to be illustrated. It should be noted that the larger transcript showed no hybridization with the *incw2* probe (data not shown).

Differential Expression of Two Sucrose Synthase Genes (*Sh1* and *Sus1*) in Response to Sugars Treatment or Depletion in Suspension-Cultured Cells

In maize suspension-cultured cells under sucrose-supplemented growth conditions, the steady-state levels of *Sh1* transcripts and the SS1 protein were relatively constant throughout one-week culture period (Figure 5-2B and 5-3B, respectively). In contrast, the steady-state levels of *Sus1* transcripts were initially reduced in the first 12 hr culture, with an increase in the levels thereafter (Figure 5-2B). SS2 protein was not significantly changed in one-week culture period (Figure 5-3B). Koch et al. (1992) observed a decrease in the steady-state levels of *Sh1* and an increase in the *Sus1* transcripts in excised maize root tips, treated with an exogenous sugar, such as 4% glucose. Consistent with this observation was the data that the *Sh1* promoter activity is repressed by the addition of higher sucrose concentration (~175 mM), and repression is released at lower sucrose concentration (~58 mM) in transient expression assay of maize protoplasts (Maas et al., 1990). In this study presented here, maize suspension-cultured cells were grown in a medium with 117 mM sucrose concentration, leading to no significant change of *Sh1* transcripts. This indicates that the sucrose concentration used

here might not be high adequately to repress *Sh1* gene expression. However, *Sus1* transcripts were initially repressed under the same sucrose concentration. It is likely that *Sus1* gene expression also can be affected by other factors, such as osmotic pressure or wounded cells due to three washes of sugar-depleted medium.

Under sugar-depleted growth conditions, *Sh1* and *Sus1* transcripts were up- and down-regulated, respectively, and such contrasting responses occurred as early as at the first 6 hr culture, followed by a nearly constant reduction until 48 hr. Nevertheless, the contrasting responses of SS1 and SS2 proteins to sugar starvation was more persistent until 48 hr (Figure 5-3A), resulting in no significant net change of total SS protein, which is also observed in maize excised root tips (Koch et al., 1992). Overall, expression of *Sh1* and *Sus1* genes in response to sugar treatment or depletion were differential in maize suspension-cultured cells and their steady-state levels of RNA transcripts were not in concert with their respective gene products at the same culture duration. Posttranscriptional control is a common feature of the regulation of the *Sh1* and *Sus1* genes (Chourey and Taliercio, 1994).

Cell division and growth under sugar starvation should be rapidly arrested. A wide variety of genes are influenced in response to this stress. In order to survive, cells have to encounter a rearrangement of stored reserves to maintain normal metabolism via sugar-modulated gene expression. In this scenario, carbohydrate reserves, such as starch grains, are digested, as evidenced by the elevated expression of α-amylase observed in rice suspension-cultured cells (Chen et al., 1994). In addition, enzymes associated with protein and lipid catabolism become more active, and autophage and total protease activity are stimulated, causing an increased in vascular volume and degradation of proteins (Chen et al., 1994; Moriyasu and Ohsumi, 1996). However, H4 histone transcripts and S27 and S28 ribosomal RNAs in maize primary roots are significantly

reduced (Chevalier et al., 1996). Such sugar-depressed ribosomal RNAs and the elevated protease activities in response to sugar availability might, at least in part, exert an effect on the function of transcriptional, translational machinery, and RNA or protein turn-over rate, leading to co-regulated or contrasting gene responses.

Finally, *Sps* transcripts or enzyme protein was low to undetectable under normal sucrose-supplemented growth conditions (data not shown). Thus, sucrose metabolism in suspension-cultured cells of maize is likely metabolized by invertase and sucrose synthase.

Figure 5-1. RNA and protein gel blots showing a tissue- or cell-specific expression of two CWI isoform genes, *incw1* and *incw2*.

(A) RNA gel blots. Total RNA was prepared from maize suspension-cultured cells (lanes, 1, 8, and 9), calli induced from immature embryo (diploid) (lanes 2 to 4), different portions of kernel (lanes 5 to 7, and 13), and 3-day germinated young shoots (lane 12) or roots (lanes 10 and 11), respectively. Each lane contained 20 μg total RNA. The RNA blots were hybridized with maize *incw1*, *incw2* or rRNA probe. SC, suspension-cultured cells; LK, UK, and WK: lower, upper, and whole kernels, respectively; R, root tips; YS, young shoots.

(B) SDS immunoblot. Crude total protein extracts were prepared from calli (the same source as mentioned above) (lanes 1 to 3), whole kernel (lane 4), and suspension-cultured cells (lane 5). Each lane contained 50 μg protein extract except lane 4, 20 μg. The blot was stained with CWI antiserum. The values showing at the right indicate the molecular mass of CWI proteins, estimated ~72 (p72) or 68 (p68) kDa, respectively.

The *incw2* transcripts and p72 polypeptide were exclusively present in kernel, whereas the *incw1* transcripts and p68 polypeptide were present in various tissues except in kernel.

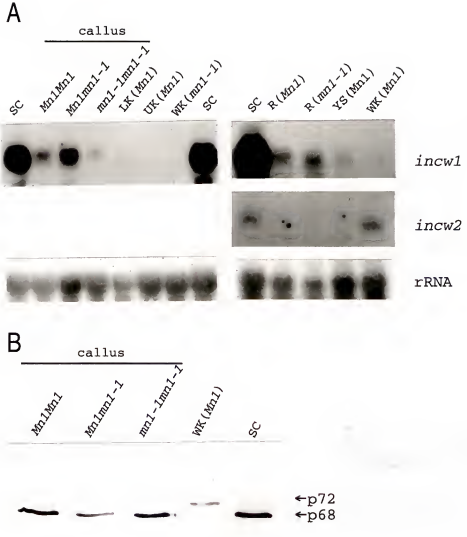

A
callus
SC
Mn1Mn1
Mn1mn1-1
mn1-1mn1-1
LK(Mn1)
UK(Mn1)
WK(mn1-1)
SC
SC
R(Mn1)
R(mn1-1)
YS(Mn1)
WK(Mn1)
incw1
incw2
rRNA
B
callus
Mn1Mn1
Mn1mn1-1
mn1-1mn1-1
WK(Mn1)
SC
←p72
←p68

Figure 5-2. RNA blots showing a differential expression of *incw1*, *Sh1*, and *Sus1* genes in suspension-cultured cells under sucrose-depleted or -supplemented treatment.

(A) Total RNA was prepared from suspension-cultured cells grown on sucrose-depleted, sucrose, or mannitol-supplemented medium.

(B) Total RNA was prepared from suspension-cultured cells grown on sucrose-supplemented medium.

(C) Total RNA was prepared from the 48 hr sugar-starved cells replenished with sucrose-supplemented medium for various periods.

The RNA blots were hybridized with maize *incw1*, *Sh1*, *Sus1* or rRNA probe. Each lane contained 20 μg total RNAs. Suc, sucrose; Man, mannitol.

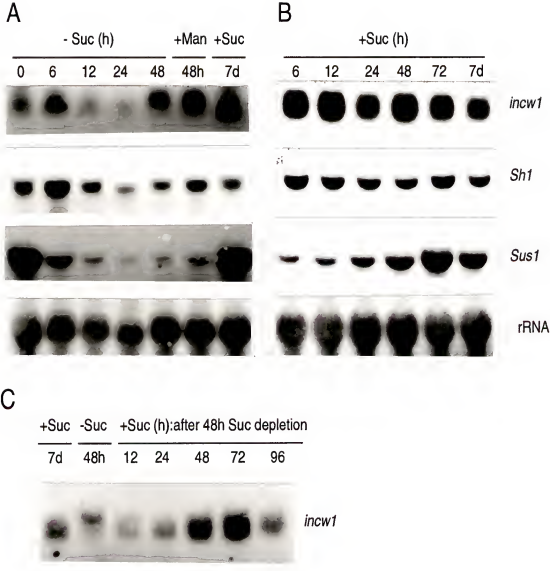

A
- Suc (h)
+Man
+Suc
0
6
12
24
48
48h
7d
B
+Suc (h)
6
12
24
48
72
7d
incw1
Sh1
Sus1
rRNA
C
+Suc
-Suc
+Suc (h):after 48h Suc depletion
7d
48h
12
24
48
72
96
incw1

Table 5-1. A Comparison of Relative Band Intensities of *incw1*, *Sh1*, and *Sus1* Transcripts in Suspension-Cultured Cells under Sugar-Starvation Stress

	-Suc					+Man	+Suc
time	0h	6h	12h	24h	48h	48h	7d
incw1	0.27	0.27	0.16	0.14	0.25	0.27	0.28
Sh1	0.21	0.38	0.18	0.11	0.12	0.16	0.12
Sus1	0.44	0.13	0.04	0.02	0.04	0.10	0.40

The quantitative values are derived from the relative band intensities of *incw1*, *Sh1*, or *Sus1* against their corresponding rRNA (see Figure 5-2) using computing densitometer scanning (see Plant Materials and Methods).
Suc, sucrose; Man, mannitol.

Table 5-2. Coordinate Regulation of Cell Wall-Bound and Soluble Forms of Invertase in Suspension-Cultured Cells

Sucrose	Enzyme Activity[a] (μmol reducing sugar/mg protein/min) Bound	(%)	Soluble	(%)	Total	(%)[b]
+12h	0.708±0.113	(100)	0.601±0.090	(100)	1.309±0.203	(100)
-12h	0.567±0.098	(80.1)	0.274±0.030	(45.6)	0.841±0.068	(64.2)
+24h	0.891±0.219	(100)	0.602±0.140	(100)	1.493±0.359	(100)
-24h	0.245±0.031	(27.5)	0.175±0.038	(29.1)	0.420±0.069	(28.1)
+72h	0.738	(100)	0.669	(100)	1.407	(100)
-72h	0.082±0.023	(11.1)	0.155±0.042	(23.2)	0.237±0.066	(16.8)

[a] Values are means ± SD of two independent experiments with the exception of +72h, each experiment with two measurements.
[b] Values within parentheses refer to its corresponding pair.

Figure 5-3. Expression of CWI-1, SS1 and SS2 proteins in suspension-cultured cells under sucrose-depleted or supplemented treatment.

Crude protein extracts were prepared from the cells with sucrose starvation or treatment at various periods. Each lane contained 50, 10, or 5 μg of crude extract for staining with CWI polyclonal, SS1, or SS2 monoclonal antiserum, respectively.

In general, the cells grown on sucrose-supplemented medium showed relatively constant of CWI-1, SS1 or SS2 protein; whereas, differential expressions of these proteins were seen when cells were grown on sugar starvation stress.

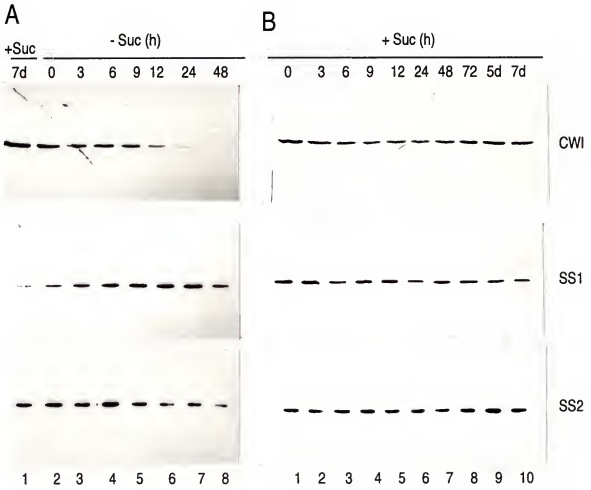
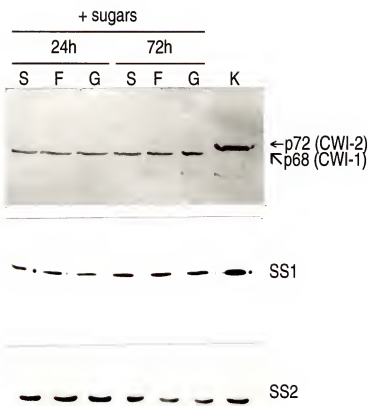

A
+Suc
- Suc (h)
7d 0 3 6 9 12 24 48
B
+ Suc (h)
0 3 6 9 12 24 48 72 5d 7d
CWI
SS1
SS2
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8 9 10

Figure 5-4. Expression of CWI-1, SS1, and SS2 proteins in suspension-cultured cells grown in various sugar-supplemented media.

Crude protein extracts were prepared from the cells treated with various sugars for 24 or 72 hr. Each lane contained 50, 2, or 10 μg of crude extracts for CWI polyclonal, SS1, or SS2 monoclonal antiserum, respectively, except lane 7 (from kernel extract) with 50, 2, or 10 μg, respectively.

In general, there was no significant change of CWI-1, SS1 or SS2 protein in each set of the same culture duration when cells were grown on various sugars for 24 or 72 hr. S, sucrose; F, Fructose; G, glucose; K, kernel.


+ sugars
24h
72h
S
F
G
S
F
G
K
←p72 (CWI-2)
↖p68 (CWI-1)
SS1
SS2

CHAPTER 6
IMMUNOLOCALIZATION OF SUCROSE-PHOSPHATE SYNTHASE IN MAIZE LEAF AND ENDOSPERM

Introduction

Sucrose is the primary and preferred form of sugar for long-distance transport from source to sink tissues in most higher plants. Sucrose-phosphate synthase (SPS; EC 2.4.1.14), a key enzyme for sucrose biosynthesis, catalyzes the conversion of UDP-glucose and fructose-6-phosphate into UDP and sucrose-phosphate. Finally, sucrose is formed after the removal of orthophosphate from sucrose-6-phosphate, catalyzed by sucrose-phosphate phosphatase (SPP). SPS is believed to control fixed carbon flux into sucrose and correlates with plant growth rate (Rocher et al., 1989; Kalt-Torres and Huber, 1987). The biochemical and kinetic properties of SPS activity/protein have been well documented (Usuda et al., 1987; Bruneau et al., 1991; Huber and Huber, 1991; Weiner et al., 1992; Reimholz et al., 1994; for review: Huber and Huber, 1996).

SPS activity shows a diurnal (day/night) fluctuation in mature leaves of maize (Sicher and Kremer, 1985). In contrast, SPS protein reveals no significant alteration under normal day/night environment (Bruneau et al., 1991). Such response is due to the effect of protein phosphorylation on this enzyme. In the light, SPS protein is activated by dephosphorylation catalyzed by a protein phosphatase (Huber and Huber, 1991). In contrast, SPS protein is inactivated through protein phosphorylation by a protein kinase during the night (Huber et al., 1989a; Siegl et al., 1990; Huber and Huber, 1991). In addition to protein modification, SPS activity is also regulated by allosteric factors, such

as glucose-6-phosphate (an activator) and Pi (an inhibitor) (Doehlert and Huber, 1983, 1985; Huber and Huber, 1992; Weiner et al., 1992).

At the molecular level, SPS has been cloned from maize (Worell et al, 1991) and spinach (Klein et al., 1993) leaves, sugar beet (Hesse et al., 1995), and rice (Sakamoto et al., 1995). The deduced amino acid sequences in these cDNA clones share a high similarity with each other (Hesse et al., 1995). In sugar beet leaf, *Sps* transcripts were up- and down-regulated by glucose and sucrose, respectively, under light condition (Hesse et al., 1995). In addition, the *Sps* promoter in rice contains a putative cis-acting element in response to light, indicating that *Sps* is probably a light-regulated gene (Sakamoto et al., 1995).

The SPS protein is abundant in mature leaves of maize (Bruneau et al., 1991; Worrell et al., 1991); however, in spinach SPS enzyme activity, protein (Walker and Huber, 1989), and RNA (Klein et al., 1993) are detected as soon as young leaves begin to expand. In addition to photosynthetic tissues, SPS is also detected in non-photosynthetic tissue in potato tuber (Klein et al., 1993; Reimholz et al., 1994) and its enzyme activity is regulated in a manner similar to the enzyme in photosynthetic tissues (Reimholz et al., 1994). SPS activity/protein and RNA are also found in seeds of *Vicia fava* (Weber et al., 1996), where it is involved in carbon partitioning. In maize, SPS RNA, protein, and enzyme activity are readily detectable in developing endosperm and restricted to cells in the lower third of basal endosperm (Chourey et al., 1993; Chourey, Cheng, Taliercio, and Im, unpublished data). SPS is also found in germinating seeds and sprouting tubers, where it is responsible for mobilizing sucrose (Hawker, 1985) which is subsequently utilized for plant growth and development.

In C4 plants, although bundle sheath (BS) and mesophyll (M) cells are metabolically coupled, the Calvin cycle reactions are unique to BS cells. Similarly,

transitory starch, a major source of carbon skeletons for sucrose synthesis during non-photosynthetic periods, is predominantly localized in BS cells of the maize leaf (Downton and Hawker, 1973; Furbank et al., 1985; Preiss, 1988). SPS is believed to play a critical role in sucrose biosynthesis through the Calvin cycle and starch turnover reactions in chloroplasts with the cytosolic reactions in both photosynthetic and non-photosynthetic environments, respectively.

Despite such compartmentalization of functions, there are conflicting reports of cellular level specificity of SPS enzyme/protein in maize leaves. Furbank et al. (1985) showed that SPS predominantly localized in the M cells, while Ohsugi and Huber (1987) demonstrated that SPS is present in both M and BS cells. However, both of these studies were based on enzyme activity in mature leaves. To better understand sucrose movement in developing endosperm, localization of SPS protein on sections of endosperm (non-photosynthetic tissue) and leaves (photosynthetic tissue) is essential. Immunohistological data shown here suggest that SPS protein is present in both M and BS cells in mature and young leaves. More importantly, there are cell-type-specific alterations in response to sink-source transition during plant development between young (sink) and mature (source) leaves. Also, such sink-source alteration is seen in light/dark treatment experiments. SPS is also present in both basal endosperm and in embryo. In addition, the data presented here show an altered pattern of developmental regulation of SPS in endosperms of *Mn1* and *mn1-1* genotypes.

Plant Materials and Methods

Plant Materials and Growth Conditions

Unless stated otherwise, all maize (*Zea mays*) lines used in this study were inbred lines of either the W22 or the Pioneer 3165 stocks. The *mn1-1* and *mn1-89* seed mutants were in W22 genetic background. The *lemon white* (*lw*), *iojap* and *mn2* mutants were in unknown inbred backgrounds. Seeds were germinated and grown in the greenhouse, growth chamber, or field; the growth temperature was maintained within 28 to 32°C in the greenhouse and at 30°C in the growth chamber. Greenhouse- or field-grown plants were under the normal diurnal (light/dark) environments, whereas plants in the growth chamber were maintained with a 12/12-hr day/night regime (irradiance 400 μmol/m^{-2} s^{-1} with incandescent and fluorescent bulbs). The leaf samples referred to here as young and mature leaves were harvested from the first or second leaves 7 to 10-day-old seedlings and the sixth to eighth leaves of 4 to 6-week-old plants, respectively. Seeds from field- or greenhouse-grown ears were harvested at various developmental stages. The harvested leaves or kernels were frozen immediately in liquid N_2 and stored at -80°C. Leaf storage was no more than 1 week prior to the immunoblot assay.

Immunolocalization in Leaf Sections

Leaf sections were fixed, embedded in paraffin, sectioned, and immunostained essentially according to the method of Langdale et al. (1987), with a few exceptions that are noted below. The first or the second leaf from seedlings, or the sixth leaf from mature plants, was cut with a razor in the midregion of the leaf into 2- to 3-mm leaf slices and fixed in formalin acetic alcohol (Jensen, 1962) with vacuum for 2 min. Prior to the selection of midregion in young leaves for our detailed analyze reported here, we examined base, middle, and tip regions of the second leaf because of the well-

demonstrated developmental gradient in young leaves (for review, see Langdale and Nelson, 1991). After fixation for 1 hr, leaf slices were dehydrated through a tertiary butyl alcohol series, infiltrated with Paraplast plus paraffin, embedded, and cut into 10-μm-thick sections using a rotary microtome. Paraffin ribbons with sections on slides were deparaffined in xylene, rehydrated to 30% ethanol, and sequentially washed in distilled water and PBS.

Immunolocalization was done by incubation in preimmune serum for the control sample, and in the polyclonal SPS antibodies against maize leaf SPS protein (Bruneau et al., 1991). The incubation time in 1:500-fold diluted antibody was adjusted to get the maximum contrast between the specific binding of the antibody to the target site and the nonspecific binding similar to that seen in the control. Slides were then incubated in a solution of secondary antibody comprised of biotinylated anti-mouse anti-rabbit immunoglobulin and streptavidin alkaline phosphatase. Visualization of the signal was done using New Fuchsia chromogen (Dako, Carpinteria, CA), which resulted in a precipitate of fuchsia-colored end product at the site of the antigen. Each comparative pair was immunostained and analyzed on the same slide to minimize possible artifacts due to staining reactions.

SDS Immunoblot Assay

Crude extracts were prepared from the frozen leaf or kernel samples by first grinding in liquid N_2, followed by homogenization using a 1:4 (young leaf) or 1:8 (mature leaf or kernel) tissue:buffer ratio in extraction buffer containing 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, and 0.1% (w/v) Triton X-100 (Huber and Huber, 1992). The homogenate was centrifuged at 14,000*g* for 1 min in a microfuge. One part of the supernatant was immediately treated with 4x SDS treatment buffer to a final volume with 1x SDS (60 mM Tris, 2% SDS, 10% glycerol, and 5%

mercaptoethanol), followed by heating on boiling water for 3 min. An aliquot was used for determining total soluble protein (Lowry et al., 1951). Denatured proteins were separated by SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. The SPS or SS polypeptides were visualized using polyclonal SPS antibodies against maize leaf SPS protein (Bruneau et al., 1991), SS1 or SS2 monoclonal antibody (Chourey et al., 1991b) and a chemiluminescence kit, following the manufacturer's protocol (Nel-100; Du Pont).

Tissue Printing

Nitrocellulose membrane (Schleicher and Schuell) was soaked in PBS buffer for 10 min, following by drying on Kimwipes. Frozen kernels at 16 DAP were cut into two parts on the embryo side and blotted onto nitrocellulose membrane for 30 seconds, following by drying at room temperature for 30 min (Cassab and Varner, 1987). The blot was then treated with the primary SPS antibody and alkaline-conjugated secondary antiserum for detection of SPS protein as described elsewhere (see: SDS immunoblot analysis section).

Results

Cell-Specific Localization of SPS Protein in Maize Leaf

Immunohistochemical analysis was done in this study to localize SPS protein in cross sections of the maize leaf. SPS protein was predominant in the BS cells of young leaf, as evidence by the intense fuchsia-colored reaction product, although a faint signal was also seen in the M cells (Figure 6-1A). In contrast, there was no detectable SPS signal in these cells in albino leaf sections (Figure 6-1B) of the *lemon white2* (*lw2*)

mutant. Albino seedlings normally die after two-week germination. Nearly equal levels of SPS signal appeared in both BS and M cells of leaf sections from 6-week-old mature plants (Figure 6-1C). The interdependence between photosynthetic ability and SPS accumulation seen in young seedlings (Figure 6-1A and -1B) was also observed in mature leaves of a 6-week-old *iojap* mutant leaf (Rhoades, 1947), characterized by pale green and albino sectors in the same leaf. There was no SPS protein detected in the cells of albino sector (Figure 6-1E, at the right end of the section), whereas SPS signal in the cells of pale sector was restricted in adaxial portion of the leaf section (Figure 6-1E, at the left end). Cells in the green sector showed normal SPS localization pattern (data not shown), as seen in Figure 6-1C. However, the preimmune section only displayed a faint and uniform background in the mature leaf (Figure 6-1F).

To further test the correlation between photosynthetic competence and SPS expression, dark-grown etiolated leaves and greening leaves by transferring the etiolated leaves to illuminated environments were examined. As expected, uniform stain constituted the background level signal in these samples (Figures 6-1G and -1J). Leaf sections from greening plant, however, showed a significant cell-specific increase in the SPS signal in BS cells, and only a lower level signal was seen in M cells (Figures 6-1H and -1I). Moreover, the increased levels of SPS signal was in a time-dependent manner. Leaf sections with 48 hr light treatment have shown more pronounced SPS signal in BS than those treated for 24 hr.

It was also examined that the effect of dark environments on light-grown plants by transferring young seedlings and mature plants to uninterrupted dark environments for 24 and 48 hr, respectively. In each case, there was a detectable reduction of SPS, particularly in M cells, and little or no change was seen in BS cells. Such spatial changes were readily seen in the leaf sections of young seedling with a 24-hr dark treatment

(Figures 6-1K versus -1L), but a similar response in mature leaves was not detectable until 48-hr dark treatment (Figures 6-1C versus -1D). At that time point, SPS signal in M cells was only slightly reduced. Although the longer duration of dark treatment, particularly 48 hr, is nonphysiological, it was done deliberately to maximize the chances of detecting specific changes, particularly at the cellular level. As pointed out by Stitt et al. (1988), regulatory mechanisms under physiological conditions might interact to generate a balance; thus, contrasting effects might not be detectable.

Spatial specificity of SPS localization in young leaves was tested by examining sections from base, middle, and tip regions of the second leaf from seedling (Figures 6-1M to -1O). A developmental gradient of the lowest and the highest SPS levels, particularly in BS cells, was seen at the base and the tip, respectively (Figures 6-1M and -1O, respectively), and intermediate levels were seen in the midregion. This is presumably reflective of the photosynthetic maturity within the leaf, as shown previously for other enzymes (Langdale et al., 1987; Langdale and Nelson, 1991). Finally, it is noteworthy that, although the SPS signal in various leaf sections appeared to be associated with plastids, we believed it is not possible to make any judgment concerning its intracellular location from these studies at the light microscope level. The observed labeling on chloroplasts may have been due to the lack of resolution of the immunocytochemical signal in this areas of cytoplasm surrounding the plastids in an otherwise vacuolated cell.

Expression of SPS Protein in Maize Leaf under Dark treatments

Figure 6-2 represents SDS immunoblots showing the effect of dark treatment on the expression of SPS protein in both young and mature leaves. Crude extracts prepared from young or mature leaves showed a single SPS polypeptide of estimated ~120 kDa (Figure 6-2). Bruneau et al.(1991) have also observed an approximate 120 kDa

polypeptide in mature leaves, but there are no such data for young leaves. Extracts from young leaves revealed a slight reduction of SPS polypeptide after 24-hr dark treatment (Figure 6-2A, lane 2). Such reduction was more pronounced after 48-hr treatment, as compared to light-grown young leaves (Figure 6-2A, lanes 3 and 1, respectively). As expected, etiolated leaves showed no detectable level of SPS polypeptide (Figure 6-2A, lane 4). Overall, the results are consistent with immunohistological data (Figures 6-1J to -1L). However, unlike young leaves, mature leaves showed no significant change in SPS levels after dark treatment for 12, 24, and 48 hr, respectively, as compared to light-grown leaves (Figure 6-2B). Although the SPS signal was slightly reduced in M cells of 48 hr dark-treated mature leaf sections (Figure 6-1D), the SPS protein was no significant change by immunoblot assay as compared to the samples without dark treatment (Figure 6-2B, lane 1 versus lane 4). It is likely that such tiny change of SPS signal in M cells can not be discriminated using immunoblot assay.

Localization of SPS Protein in Developing Endosperm Using Immunoblot and Tissue Printing Analyses

Northern blot analyses have shown that *Sps* transcripts are localized entirely in the basal part of a developing endosperm (Taliercio and Chourey, unpublished data). To further determine the location of SPS protein in the developing kernel, both SDS immunoblot and tissue printing methods were used in this study. The SDS immunoblot shown in Figure 6-3 revealed that SPS protein was exclusively restricted to the lower third of endosperm (Figure 6-3A, lane 3); in contrast, the protein was undetectable in the upper parts of endosperm (Figure 6-3A, lane 2). The protein in both mature leaf (Figure 6-3A, lane 1) and endosperm appeared to be the same size, approximately 120 kDa. The mixture obtained from equal amounts of upper and lower parts of endosperm extracts

revealed a reduced band intensity (Figure 6-3A, lane 4), consistent with the preferential location of SPS in the lower part of endosperm (Figure 6-3A, lane 3).

We have also attempted to immunolocalize SPS protein on tissue sections from fixed kernels at the cellular level, but these efforts have been unsuccessful. Since SPS is a relatively labile protein (Huber, 1996), or it is likely that the SPS epitope sites are buried or destroyed after FAA fixation. To bypass the fixation process, an alternative method, tissue printing, was used in this study (see Plant Materials and Methods). The positive signals due to the SPS protein, as judged by an intense black color, were limited to the basal part of the endosperm. A low level of staining was also seen on the embryo (Figure 6-3B, two prints on the left). In contrast, the preimmune-treated control only showed a relatively faint background (Figure 6-3B, two prints on the right).

Expression of SPS protein in Developing Endosperm of the *mn1-1* Seed Mutant

All *mn1* seed mutants examined thus far revealed either considerably low (e.g., *mn1-89*) or undetectable (e.g., *mn1-1* and other EMS-induced mutants) levels of invertase activity and the CWI protein. Also, invertase is the first sucrose-metabolizing enzyme that metabolizes the incoming sucrose in developing kernel and plays a critical role in normal development of endosperm and maternal cells in the pedicel. SDS immunoblot analyses were conducted to examine the levels of SPS and SS proteins to test if invertase-deficiency has also affected these enzymes.

Figure 6-4 represents the levels of SPS and SS proteins in various seed mutants. Band intensity of SPS protein in the *mn1-89* mutant was similar to that in homozygous *Mn1* wild type (Figure 6-4A, lanes 3 and 4, respectively); however, the *mn1-1* mutant showed a slightly elevated level of SPS protein when compared to the *Mn1* wild type at 16 DAP (Figure 6-4A, lanes 2 and 4, respectively). As for the two SS isoforms, in

general, abundance of the SS1 and SS2 proteins were unchanged in the *mn1-89* mutant as compared to the wild type (Figure 6-4A, lanes 3 and 4, respectively). In contrast, a significant reduction of both SS isoforms was seen in the *mn1-1* mutant (Figure 6-4A, lane 2). Another non-allelic miniature seed mutant, *mn2*, revealed nearly normal levels of SPS and SS proteins, as compared to wild type (Figure 6-4A, lanes 1 and 4, respectively).

To further determine whether such a reduction of SS and increase of SPS in the *mn1-1* mutant also occur at various stages, immunoblot assays were used to detect SS and SPS proteins throughout kernel development. As shown in Figure 6-4B, the highest level of SPS protein in homozygous *Mn1* genotype appeared at 12 to 16 DAP (Figure 6-4B, lanes 2 and 3), with a rapid reduction thereafter (Figure 6-4B, lanes 4 to 7). The levels of the SPS protein remained relatively constant during 20 to 28 DAP. Overall, this SPS expression pattern in developing endosperm is similar to the developmental profile of invertase activity and protein (Figure 3-1). In contrast, although SPS protein in the *mn1-1* seed mutant remained relatively constant (Figure 6-4B, lanes 8 to 10) from an early developmental stage (12 DAP) to a later stage (24 DAP), the protein levels, as judged by band intensity, were higher than those in the wild type at the corresponding stages, particularly at 24 DAP.

For sucrose synthase, the highest amount of the two isoforms (SS1 and SS2) occurred approximately 20 to 24 DAP (Figure 6-4B, lanes 4 and 5), with a reduction thereafter. Both SS1 and SS2 isozymes were at much reduced levels in the *mn1-1* mutant during 12 to 24 DAP (Figure 6-4B, lanes 8 to 10) as compared to wild type at the same developmental stages.

Discussion

Altered Expression at the Cellular Level by Developmental Transition and by Photoregulation

The data presented here are important, particularly for the observations concerning the cellular specificity of SPS protein and the correlated alteration in its expression due to development at transition (sink to source) and photoregulation.

The immunohistological data showed that SPS protein was prevalent in both BS and M cells in mature leaves, consistent with the enzyme activity reported previously (Ohsugi and Huber, 1987). In contrast, Furbank et al.(1985) observed that SPS activity was ~10-fold higher in M than that in BS cells. However, there is a large range of variability in these estimates, and the lowest ratio of M:BS was 2.0. It is possible that the time lag involved in the fractionation of the two cell types could account for such variability. The *in situ* analysis used in this study, although not quantifiable, did not encounter such a delay due to the rapid fixation of leaf sections. Moreover, to date there are no SPS data showing entire heterotrophy in young leaves (Deleens et al., 1984). Here, our studies of such sucrose-importing leaves have shown predominant localization of the SPS protein in BS cells. Similar results were also observed when greening leaves obtained by the transfer of dark-grown etiolated seedling to normal light condition generated a strong SPS signal in BS cells. There was no SPS protein detectable in leaf sections from either the albino seedlings of the *lemon white* mutant or the etiolated seedlings. It might reflect that the SPS protein in BS cells of the sucrose-importing leaves was either dependent on or correlated with the photosynthetic competence of these cells.

Light is well documented to serve as a major signal in the control of BS-specific expression of the ribulose-1,5-bisphosphate carboxylase gene (Sheen and Bogorad, 1987;

Langdale et al., 1988), and the enzymes in the Calvin cycle pathway (Hatch and Osmond, 1976). In the long chain of photoassimilatory reactions, SPS is an important cytosolic enzyme controlling the flux of carbon fixation into sucrose. Photosynthetic metabolites, including sucrose, have been reported to regulate the expression of a number of plant gene (Sheen, 1990). Wang et al. (1993) also suggested that accumulation and transport of photoassimilates may influence developmental patterns of gene expression, since the cell-type-specific changes in ribulose-1,5-bisphosphate carboxylase expression are in coordination with sink to source transition in amaranth leaves. The co-localization of SPS protein with the Calvin cycle enzymes in BS cells, especially in greening leaves and in young leaves from light-grown seedlings suggest that SPS in BS cells may play a major role in the early biosynthesis of sucrose in developing leaves. It is possible that photosynthetic competence in terms of carbon fixation into sucrose in BS cells precedes that of M cells; the former might export sucrose to the latter for a brief time in young leaves.

The dark treatments also produced a differential cellular response in BS and M cells in SPS expression. Although there was no significant detectable change in the signal level in BS cells, the levels in M cells were appreciably reduced; such reductions were much more rapid in young leaves than in mature leaves. Ohsugi and Huber (1987) observed a similar high percentage of whole-leaf SPS activity in BS cells early in dark periods. We suggest that the cell-specific response in M cells is related to the predominant role these cells play in the photoassimilatory biosynthesis of sucrose. In dark environments, the depleted pools of sucrose may down-regulate SPS expression in M cells, which has been shown previously in enzyme activity determinations at the whole-plant level under the diurnal control of growth environment (Furbank et al., 1985; Ohsugi and Huber, 1987; Stitt et al., 1988). In contrast, the relative insensitivity of SPS

protein in BS cells to dark environments may indicate that it is less sensitive to light and is also responsible for non-photosynthetic biosynthesis of sucrose through starch turnover (Ohsugi and Huber, 1987; Farrar, 1991), since starch is strictly confined to BS cells in maize leaves (Downton and Hawker, 1973; Furbank et al., 1985; Preiss, 1988). Thus BS cells may again serve as a sucrose source during the non-photosynthetic environments.

Expression of SPS and a Possible Route of Sucrose Transport in Developing Kernel

Sucrose-metabolizing enzymes (SS, SPS, and/or invertase) in most higher plants examined thus far, are normally present together in the same sink tissues (Geigenberger and Stitt, 1991) to metabolize sucrose. In the developing kernel, for instance, invertase was present along the terminal ends (or upper parts) of vascular bundles of the pedicel (Figures 3-5B and -5C) and predominant in the extracellular space of basal endosperm transfer cells (Figures 3-5B to -5C). Conversely, the SPS protein was found exclusively within the basal endosperm cells and cells of the embryo (Figure 6-3B). Moreover, developmental profiles of SPS (Figure 6-4) paralleled that of the invertase (Figure 3-1); the highest levels of SPS were seen during the period from 12 to 16 DAP. These data suggest a model of extracellular sucrose hydrolysis and intracellular resynthesis in the basal endosperm cells likely occurring in a coordinate manner. The differential expression of SS isoenzymes, SS1 and SS2, in developing endosperm has been previously well documented (Chen and Chourey, 1989; Rowland and Chourey, 1990; Heinlein and Starlinger, 1989). The SS2 isoform is abundant in embryo, and the lower and middle portions of endosperm. SS2 protein is almost undetectable in the upper portion of endosperm, as evidenced by immunolocalization analyses (Chen and Chourey, 1989; Heinlein and Starlinger, 1989). Conversely, SS1 was restricted to the upper part of endosperm with a gradual decrease toward the basal region. Thus, the SS1 enzyme was

low to undetectable in the lower third of endosperm, which is mainly composed of basal endosperm transfer cells and the elongated cells.

A possible route for sucrose transport in developing kernel is illustrated based on the location of the sucrose-metabolizing enzymes. In developing kernel, sucrose arriving at the terminal ends (or upper parts) of the vascular bundles of the pedicel must be unloaded through the apoplastic path, since there are no plasmodesmata connections between cells in the placento-chalazal region and the endosperm. Among other factors, the CWI along the terminal ends of vascular bundles is believed to function as a "reflux valve", which may prevent sucrose from reloading by hydrolysis of sucrose into fructose and glucose (Eschrich, 1980). Thus, a large proportion of sucrose along with fructose and glucose, are apoplastically fluxed into the extracellular space of basal endosperm transfer cells, where sucrose is presumably hydrolyzed by CWI and taken up by basal endosperm transfer cells in a passive-force manner (Porter et al., 1985; Griffith et al., 1987, Felker 1992). Subsequently, the hexose sugars in the basal endosperm cells may be resynthesized to form sucrose, which in turn is transported to the upper part of endosperm, where sucrose is metabolized and utilized mainly for starch and cell wall biosyntheses.

Overall, the hydrolysis of sucrose within the apoplast of kernel base (the pedicel and basal endosperm transfer cells) might serve to maintain a steep sucrose concentration gradient from source to sink tissues (Miller and Chourey, 1992). The maintenance of such a gradient may be strengthened further by the rapid resynthesis and removal of sucrose from transfer cells to the upper part of endosperm, where it is utilized for starch or cell wall biosyntheses. The sucrose resynthesis and utilization in endosperm are mainly through SPS and SS activities.

SPS Protein Levels in the *mn1-1* Seed Mutant

A comparison of SPS and SS proteins among the *mn1* seed mutants (*mn1-1* and *mn1-89*) and wild type revealed that approximately 6% of total invertase activity, as seen in *mn1-89* mutant, resulted in nearly normal levels of SPS and SS proteins in developing kernel at 16 DAP (Figure 6-4). In contrast, the levels of SPS and SS proteins in the *mn1-1* mutant varied developmentally when compared to the wild type (Figure 6-4). The SS isoforms, SS1 and SS2, in the *mn1-1* mutant declined significantly during 12 to 24 DAP. However, SPS levels were increased during the same developmental stages. Such contrasting gene-expression patterns of SS and SPS proteins in the *mn1-1* mutant as compared to the wild type, can not be explained by the influence of the maternal genotype since the *mn1-1* seed mutant segregating from the normal wild-type seeds in segregating ears also showed similar patterns for the SS and SPS proteins.

We are unaware of any SPS mutants. Thus, physiological significance of SPS protein in developing endosperm remains to be understood. Similarly, it is not clear at present why the SPS protein levels in the *mn1-1* mutant are higher than the *Mn1* kernels.

Figure 6-1. Immunolocalization of SPS in leaves from various samples.

(A) Young green leaf, *Lw* genotype, grown under the normal diurnal light/dark cycle.

(B) Young albino leaf, *lw* genotype; the same growth environment as in (A).

(C) Mature leaf under the normal diurnal light/dark cycle.

(D) Mature leaf; the same growth environment as in (C) plus 48 hr of continuous dark treatment.

(E) Mature leaf, *iojap* mutant; the section represents pale-green and albino sectors; cells representing the albino sector are at the right.

(F) Mature leaf as in (C) but treated with preimmune serum. E, epidermal cells; M, mesophyll cells; BS, bundle sheath cells; V, vascular bundle.

Magnification is X59.4 except in (E), X29.7.

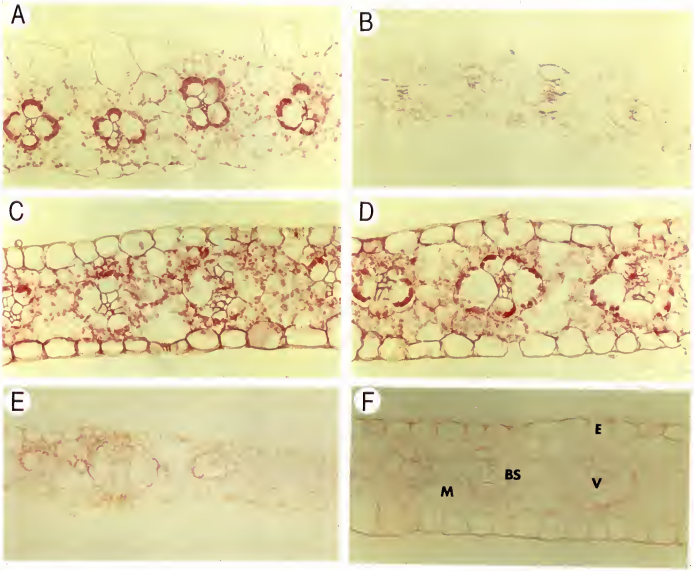

A
B
C
D
E
F
E
BS
V
M

Figure 6-1. (Continued) Immunolocalization of SPS in leaves from various samples.

(G) Etiolated young leaf from dark-grown seedlings.

(H) Leaf from dark-grown seedling plus a 24-hr normal diurnal condition in the greenhouse.

(I) Leaf from dark-grown seedlings plus a 48-hr normal diurnal condition in the greenhouse.

(J) Etiolated young leaf.

(K) Young green leaf from seedlings grown under the normal diurnal light/dark cycle.

(L) Young green leaf; the same as in (K) plus 24 hr of continuous dark treatment.

(M), (N), and (O) Young green leaf from seedling grown under normal diurnal light/dark cycle, representing sections from base (M), middle (N), and tip (O) portions of second leaf.

Magnification from (G) to (O) is X59.4.

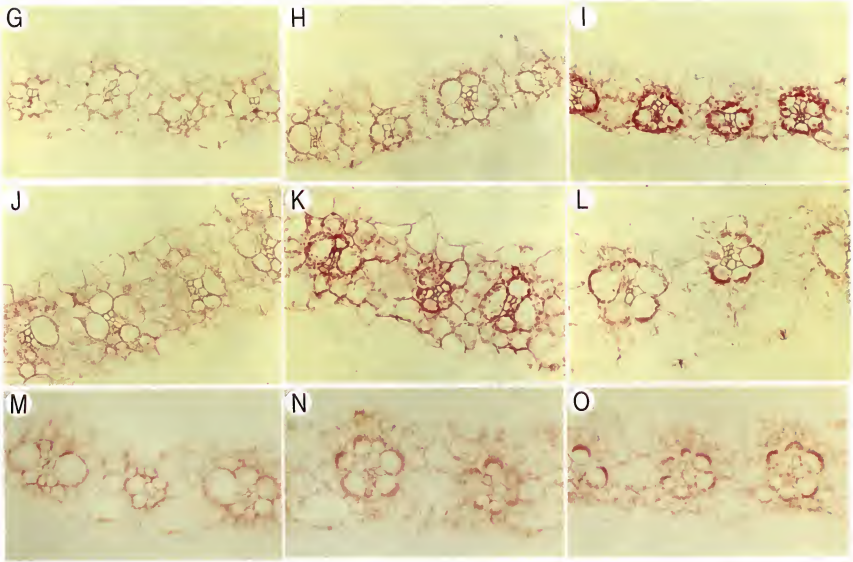
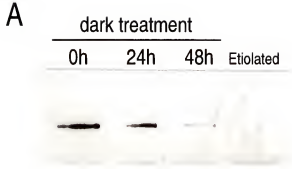

G
H
I
J
K
L
M
N
O

Figure 6-2. SDS immunoblots showing SPS protein in young and mature leaves under dark treatments.

(A) SDS immunoblot analysis showed SPS protein in extracts from young leaves (8-day-old) under dark treatments and etiolated leaves. Each lane contained 100 μg crude extract.

(B) SDS immunoblot analysis showed SPS protein in extracts from mature leaves (6-week-old) under dark treatments. Each lane contained 100 μg crude extract.


A
dark treatment
0h
24h
48h
Etiolated

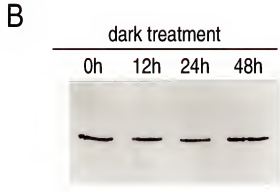

B
dark treatment
0h
12h
24h
48h

Figure 6-3. Localization of SPS protein in developing kernel.

(A) SDS immunoblot showing SPS protein in developing kernel. Total crude protein extracts were prepared from mature leaf (lane 1), upper two-thirds (lane 2), lower third (lane 3), and the mixture of equal amounts of upper and lower extracts of kernel without embryo (lane 4) at 16 DAP. The extract of upper kernel on lane 5 was cut out and stained with SS1 monoclonal antiserum. Each lane (from lanes 1 to 5) contained 50, 100, 100, 100, and 10 μg crude extracts, respectively.

(B) Tissue printing of SPS protein in developing kernel. Frozen kernels at 16 DAP were cut into two parts and pressed lightly on nitrocellulose membrane, followed by protein gel blot analysis. Polyclonal SPS antiserum was used in this study (two prints at the left); preimmune was used as a negative control (two prints at the right).

SPS protein was localized to the lower third of developing endosperm (A), particularly, restricted to basal endosperm cells and embryo (B).

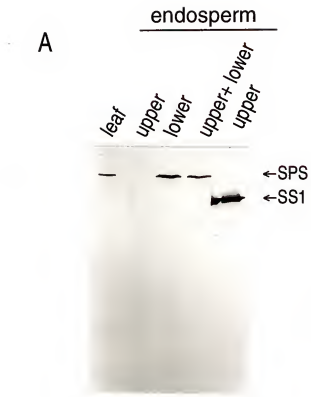
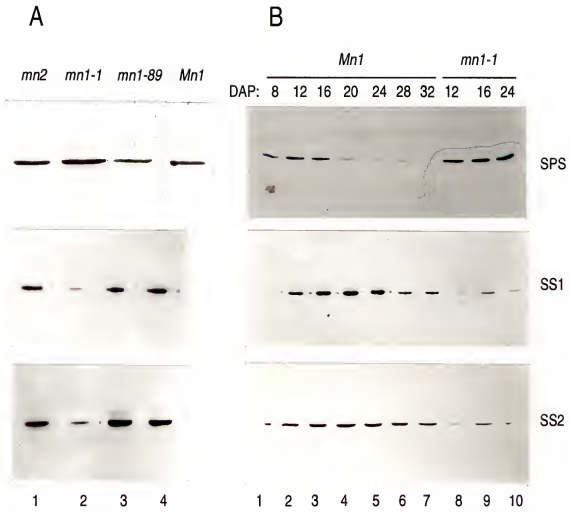

endosperm
A
leaf
upper
lower
upper+ lower
upper
←SPS
←SS1
B
tissue printing

Figure 6-4. SDS immunoblots showing the expression of SPS and sucrose synthase proteins in various seed mutants and at various developmental stages.

(A) Crude protein extracts were prepared from kernels at 16 DAP with homozygous *mn2*, *mn1-1*, *mn1-89*, and *Mn1* genotypes. Each lane contained 100, 2, or 10 μg crude protein extract for reactions with SPS polyclonal, SS1, or SS2 monoclonal antiserum, respectively.

(B) Crude protein extracts were prepared from kernels at various developmental stages with homozygous *Mn1* or *mn1-1* genotype. The amount of crude protein extracts in each lane and the treatments of anti-sera were the same as (A).

Overall, SPS and SS proteins in the *mn1-1* seed mutants were up- and down-regulated, respectively, but nearly wild-type levels of these proteins were seen in the *mn2* and *mn1-89* seed mutants at 16 DAP.


A
mn2
mn1-1
mn1-89
Mn1
1
2
3
4
B
Mn1
mn1-1
DAP: 8 12 16 20 24 28 32 12 16 24
SPS
SS1
SS2
1 2 3 4 5 6 7 8 9 10

CHAPTER 7
SUMMARY

Collective evidence supports a hypothesis that the *Miniature1* (*Mn1*) seed locus encodes a cell wall-bound invertase (CWI), CWI-2. First, CWI activities and/or proteins revealed a gene-dose relationship with the copy number of *Mn1* (or *mn1-89*) allele in three sets of parents and their reciprocal hybrids, *Mn1/mn1-1*, *Mn1/mn1-89*, and *mn1-89/mn1-1*. In each set of experiments, kernels with three copies of *Mn1* (or *mn1-89*) allele showed the highest levels of CWI specific activity or protein, with a gradual reduction in two, one and zero copies of *Mn1* (or *mn1-89*) allele. Second, RFLP mapping showed that the *incw2* cDNA clone, isolated from immature kernel base (Taliercio et al., 1995), mapped to chromosome 2L and was inseparable from the *mn1* locus in genetic mapping (Coe et al., 1995; Chourey, personal communication). Third, The *Mn1* seed locus exhibited a co-segregation with the *incw2* gene in F_2 or advanced generations (Chourey and Taliercio, personal communication). Finally, The *incw2* cDNA discriminated various *mn1* mutants into two groups. One group had detectable levels of *incw2* transcripts, but the other did not (Chourey and Taliercio, personal communication). Furthermore, the former group was divided into two subclasses. One subclass had detectable CWI-2 protein, but the other did not. Taken together, the *Mn1* seed locus is a structure gene which encodes *incw2* RNA and CWI-2 (p72) polypeptides as the protein.

Unlike the invertase-deficient *mn1-1* seed mutant with a ~70% loss of wild-type seed weight and a gap formation between pedicel and endosperm, the *mn1-89* seed

mutant showed ~6% total invertase activity of the wild type, nearly normal seed size/ weight, and no gap formation between pedicel and endosperm. Furthermore, the hybrids with two copies of *mn1-89* allele, obtained by reciprocal crosses between *mn1-89mn1-89* (as female parent) and *mn1-1mn1-1* parents, had ~4% total invertase activity of the wild type and a slightly reduced seed size/weight as compared to its parent homozygous *mn1-89*, and there was no gap formation between pedicel and endosperm. In contrast, the hybrids with one copy of *mn1-89*, obtained by the reciprocal cross *mn1-1mn1-1* (as female parent) with *mn1-89mn1-89*, had ~2% total invertase activity of the wild type, a seed phenotype indistinguishable from its parent homozygous *mn1-1*, and a gap formation between pedicel and endosperm. Thus, a substantial level of invertase activity (~90%) is redundant, but there is a threshold value (~10%), below which the invertase plays a rate-limiting role in normal development of endosperm and the stability of cells in the pedicel.

Both cell wall-bound and soluble forms of invertase activities revealed a coordinate regulation throughout kernel development in various genotypes tested. Developmental profiles of invertase specific activity in endosperm showed that CWI is the predominant form (~90%), whereas the soluble form of invertase was only ~10% of total invertase activity. Moreover, a gene-dose relationship of the *Mn1* (or *mn1-89*) allele with enzyme activity in various genotypes showed that the increase or decrease of CWI activity was always associated with the corresponding change of activity in soluble form.

Immunolocalization data showed that CWI protein in the homozygous *Mn1* kernel sections was predominantly localized in the two cell layers of basal endosperm transfer cells (BETC), and a low level CWI signal was also detectable in the pedicel along with upper parts of vascular bundles. As expected, CWI protein was low to undetectable in these regions in both *mn1-1* and *mn1-89* seed mutants, which only had ~1.5 and 6.0% total invertase activities, respectively. Consistent data were observed in *in-situ*

hybridization with labeled-*incw2* RNAs. The *incw2* transcripts were readily detectable in the two cell layers of BETC in *Mn1* kernel sections hybridized with *incw2* antisense RNAs. However, unlike immunolocalization, the *incw2* transcripts were low to undetectable in the pedicel of *Mn1* kernel sections, presumably due to the low detection limits of this method.

Specific activity levels for invertase in homozygous *Mn1* kernels, grown *in vitro* on various sugar-supplemented media, were reduced by ~20 to 30% as compared to the kernels grown *in planta*. The diminished levels of invertase activity were limited to CWI, consistent with the immunolocalization data showing that the CWI protein was localized only in a single cell layer of BETC, rather than two cell layers of BETC as seen in kernels grown *in planta*. The early withdrawal of pedicel from endosperm, i.e., a gap formation in the *mn1-1* seed mutant, is due to the loss of invertase activity in kernel base of this mutant (Miller and Chourey, 1992). The *mn1-1* developing kernels retained the *mn1-1* seed mutant phenotypes and a gap between pedicel and endosperm, although they were grown on reducing sugars. In addition, crude extracts from cob tissue which support *in vitro* culture of kernels were examined for invertase, SS isoenzymes and SPS by immunoblot analyses. Although there was no invertase activity/protein, the last two enzymes were detectable. Possible significance of SS and SPS in cob tissue remains to be understood.

In maize suspension-cultured cells, both cell wall-bound and soluble forms of invertase activities also revealed a coordinate control under sugar starvation. Moreover, CWI-1 (or p68) polypeptide was reduced in a time-dependent manner under the same growth conditions, consistent with the reduction of CWI activity. In contrast, CWI-1 protein was relatively constant throughout one-week culture period under sugar-supplemented medium. At the molecular level, the steady-state levels of the *incw1*

transcripts in cultured cells showed no significant change during one-week culture period under sucrose-supplemented medium. However, the steady-state levels of the *incw1* transcripts were reduced after 12 hr culture under sugar starvation. Surprisingly, another new larger *incw1* transcript was detectable on the blot with total RNA extracts from 48 hr sugar-starved or mannitol-cultured cells. Moreover, the new larger *incw1* transcript was untranslated based on our SDS immunoblot and enzyme activity assays. The molecular basis of this larger transcript is unknown.

As for expression of sucrose-phosphate synthase (SPS) in maize leaf and kernel, immunohistological analyses for SPS showed that the protein was localized in both bundle sheath (BS) cells and mesophyll (M) cells in maize leaves. In young leaves, SPS was predominant in the BS cells; whereas, in mature leaves it showed nearly equal levels of SPS signal in both BS and M cells. A cell-type-specific response was also seen in light and dark treatments. Dark treatments for 24 or 48 hr led to a reduced levels of SPS signal in M cells, particularly in young leaves; however, little or no change was detected in BS cells. These cell-specific changes were in agreement with the corresponding levels of SPS protein under the same growth conditions. In greening experiments by transferring etiolated seedling to normal diurnal day/night environments, a significant level of SPS signal was induced in BS cells. Taken together, we suggest that SPS in BS cells is engaged in sucrose biosynthesis by means of both photosynthetic and starch turn-over reactions. In addition, the enzyme in BS cells may play a major role in the early biosynthesis of sucrose in young leaves.

SPS protein in developing endosperm, a nonphotosynthetic tissue, was localized to the basal endosperm cells and embryo using *in situ* tissue printing method. Because spatial and temporal profiles of SPS and invertases in developing endosperm were in parallel, it is possible that sucrose breakdown and resynthesis occur prior to the proper

utilization of sucrose. Interestingly, SPS protein in the invertase-deficient *mn1-1* seed mutant was up-regulated throughout the entire development of the kernel. The physiological significance of the elevated levels of the SPS protein in this mutant is unknown.

APPENDIX

FAA Fixative

95% ethanol alcohol	50 ml
glacial acetic acid	5 ml
37% formaldehyde	10 ml
destilled water	35 ml

TBA Dehydration Series

Step	95% EtOH	TBA	H_2O	Time
	ml	ml	ml	day
I	40	10	50	1
II	50	20	30	1
III	50	35	15	1
IV	45	55	0	1.5
V	25*	75	0	1.5
VI	0	100	0	1
VII	0	100**	0	1
VIII	0	100	0	1

*: 100% ethanol alcohol. **: Safranin O added.

Kernel Culture Medium

Compound	Stock Con.	Final Con.
Major element I	50X	
Major element II	100X	
Minor elements	100X	
Fe-EDTA	2000X	0.5 ml/L
Niacin	1000X	1.2 ml/L
Thiamin	1000X	0.4 ml/L
Folic acid	100X	0.044 ml/L
Glutamine		2.2 g/L
Asparagine		2.0 g/L
Lysine		183 mg/L
Casein hydrolysate		1.0 g/L
pH to 5.8		
Agar		5.5 g/L
2,4-D	100X	1.0 mg/L

Add 300 ml distilled water into 500 ml beaker or flask, then sequentially add stock solutions. Bring total volume to 400 ml, adjust pH to 5.8, add agar, and then autoclave for 15 min. The autoclaved medium was cooled down to 50°C and add the followings by filter sterilization (the final value ~500ml):

Streptomycin sulfate	1 g/5 ml	0.5 ml/L
Carbohydrate (Suc, Fru, or Glc)		15%

Components of Stock Solutions

	Final Conc. (mg/L)
Major element I: 50X	
KNO_3	2,500
NH_4NO_3	165
$CaCl_2.2H_2O$	176
Major element II: 100X	
KH_2PO_4	510
$MgSO_4.7H_2O$	370
Fe-EDTA: 0.1 M solution	
Na_2EDTA	18.6
$FeSO_4.7H_2O$	13.9
Minor elements: 100X	
$MnSO_4.H_2O$	16.9
H_3BO_3	6.2
$ZnSO_4.7H_2O$	2.87
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025

Medium for Callus Induction or Cell Suspension Culture

Compound	Stock	Final Conc
Major element I	50X	see above
Major element II	100X	"
Minor elements	100X	"
Fe-EDTA	2000X	"
Asparagine		0.169 g/L
Carbohydrate (sucrose)		2 or 3 g/L
2,4-D	100X	2 or 4 mg/L
pH 5.8		

The medium for callus induction contained sucrose 3 g/L and 2,4-D 2 mg/L, whereas it contained 2 g/L and 2,4-D 4 mg/L for cell suspension culture.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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